

***In Situ* Bioremediation and Natural Attenuation of  
Dinitrotoluenes and Trinitrotoluene**

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By

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***In Situ* Bioremediation and Natural Attenuation of  
Dinitrotoluenes and Trinitrotoluene**

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This thesis is lovingly dedicated to my wife, JUNG-A CHO

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## NOMENCLATURE

### Chemical Compounds

ACN	Acetonitrile
DNT	Dinitrotoluene
2,4-DNT	2,4-dinitrotoluene
2,6-DNT	2,6-dinitrotoluene
NT	Nitrotoluene
TNT	2,4,6-Trinitrotoluene
HNOHA	2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid
HNPA	2-hydroxy-5-nitropenta-2,4-dienoic acid
ADNT	Aminodinitrotoluene
DANT	Diaminonitrotoluene
TAT	2,4,6-triaminotoluene
SN	Sum of Nitrogen ( $\text{NO}_2^-$ and $\text{NO}_3^-$ )

### Biological and Engineering Notation

R	Overall reaction
$R_{\text{ea}}$	Half-reactions for the electron acceptor
$R_{\text{ed}}$	Half-reactions for the electron donor
$R_{\text{c}}$	Half-reactions for the cell material
$f_{\text{c}}$	Fractions of electrons used for energy generation
$f_{\text{s}}$	Fractions of electrons used for cell synthesis
$\Delta G_{\text{r}}$	Free energy released per equivalent of donor oxidized for energy-generation (kcal energy available/eq.electron donor used for energy)

$G_f^\circ$	Standard Gibbs free energy
$\Delta G_n$	Energy required by bacteria to convert nitrogen source to $\text{NH}_3$ (kcal/eq.)
$\Delta G_p$	Energy required by bacteria to convert nitrogen source to pyruvate (kcal/eq.)
$\Delta G_c$	Energy required by bacteria to convert pyruvate and $\text{NH}_3$ to cellular material (kcal/eq.)
$\Delta G_s$	Energy required for cell synthesis (kcal/eq. cells synthesized)
$\varepsilon$	Bacterial metabolic efficiency
DO	Dissolved Oxygen
COD	Chemical Oxygen Demand
HRT ( $\theta$ )	Hydraulic Retention Time ( $\text{d}^{-1}$ )
$S_{\min}$	Minimum Substrate Concentration
X	Biomass Concentration ( $\mu\text{M}$ )
S	Substrate Concentration ( $\mu\text{M}$ )
Y	Yield Coefficient ( $\mu\text{mol X}/\mu\text{mol S}$ )
k	Maximum Specific Growth Rate ( $\mu\text{mol S}/\mu\text{mol X-d}$ )
$K_s$	Half-Velocity Constant ( $\mu\text{mol S/L}$ )
b	Bacterial Decay Coefficient ( $\text{d}^{-1}$ )
CSTR	Continuously Stirred Tank Reactor
v	Pore Water Velocity (cm/min)
D	Dispersion coefficient ( $\text{cm}^2/\text{min}$ )
n	Porosity
R	Retardation Factor
HS	Highly DNT Contaminated Soil

MS	Medium DNT Contaminated Soil
LS	Low DNT Contaminated Soil
N-ratio	Ratio of Nitrate to Nitrite Evolved
DNR	Denitrification
rt	Retention Time
$\lambda$	Wave Length (nm)
<u>Other</u>	
NA	Natural Attenuation
MNA	Monitored Natural Attenuation
WI	Wisconsin
VAAP	Volunteer Army Ammunition Plant
BAAP	Badger Army Ammunition Plant
AFRL	Air Force Research Laboratory
EPA	Environmental Protection Agency
CFR	Code of federal regulation (40 CFR 268.48)
HPLC	High-Performance Liquid Chromatography
IC	Ion Chromatography
GC	Gas Chromatography
MS	Mass Selective Detector
UV	Ultra-Violet
LSC	Liquid Scintillation Counter

## SUMMARY

Two isomers of DNT, specifically 2,4-DNT, and 2,6-DNT, exist as soil and groundwater contaminants at former TNT production sites and in the wastewater from the commercial production of feedstocks for polyurethane foam. The discovery of bacteria that use DNT isomers as energy, carbon, and nitrogen sources has encouraged the potential application of bioremediation at contaminated sites. This work focuses on extending the existing ex-situ engineered bioremediation to *in situ* biodegradation and focuses on the application of in place soil bioremediation and *in situ* natural attenuation (NA) as remediation strategies for treating DNT at contaminated sites.

More specifically, this research evaluated factors influencing *in situ* bioremediation of DNTs and TNT in surface soils, vadose zones, and saturated media. Applications involving surface soils and vadose zones investigated the potential of water infiltration to promote *in situ* bioremediation. Studies in saturated media were more applicable to NA. Factors that were also considered in studies conducted included: 1) the presence and distribution of degrading microbes in field soils; 2) the dissolution and bioavailability of contaminants in historically contaminated soils; and 3) the effect of mixtures of contaminants on biodegradation processes. In the following sections, the findings regarding each of these factors are presented, along with areas where increased understanding would remove bottlenecks to implementation within field sites.

Initial biodegradation tests were carried out using soils from a historically contaminated DNT site (*i.e.*, Barksdale, WI). Microcosms and columns constructed with soil and water from the sites showed that indigenous biological activity was responsible

for the destruction of DNT. In column systems rapid DNT destruction occurred, but limited 2,6-DNT mineralization was observed in the presence of 2,4-DNT. Evolved N-ratio (*i.e.*,  $\text{NO}_3^-/\text{NO}_2^-$  ratio) was dependent upon the mass of DNT remaining in soils, indicating that the N-ratio may be useful monitoring tool for DNT depletion.

Subsequent studies assessed how degradation by-products (nitrite) and environmental factors (pH) influence the biodegradation of DNT leaching into vadose zones. The column experiments demonstrated that the degradation of DNT isomers was severely inhibited at or below pH 5.5. The presence of nitrifying bacteria diminished the sensitivity to nitrite by converting it to nitrate. 2,4-DNT degradation decreased at 70 mM of nitrite in feed solution and no decrease of 2,6-DNT degradation in the presence of nitrite (10 to 130 mM) was observed. Hydraulic retention time (HRT) had a significant effect on effluent DNT concentrations, while enhanced aeration had only a slight influence.

Subsequently, pilot scale studies were conducted to assess the scale-up of infiltration based bioremediation processes for DNT contaminated soils (referred to as DNT BioPilot study). The study focused on evaluating the rate and extent of DNTs biodegradation in the soils and underlying vadose zone under simulated *in situ* bioremediation conditions involving water infiltration. Results from this study indicate that 2,4-DNT and 2,6-DNT were rapidly biodegraded by naturally existing microorganisms in the soil pilot system. The presence of added COD negatively influenced the degradation of 2,6-DNT in the soil.

The NA of DNT plumes were evaluated to understand the minimal concentration limits for growth on either 2,4-DNT or 2,6-DNT. Experiments were conducted using

chemostats and glass bead columns seeded with DNT degrading cultures. The minimum achievable values for DNT isomers were much lower than the values recommended by the US EPA and the CFR and suggest that NA, with active biodegradation, has the potential as an effective remediation strategy of DNT contaminated plumes if aerobic conditions exist.

There are areas contaminated with mixtures of TNT and DNT isomers at TNT manufacturing sites. Previous studies have shown that TNT can be cometabolized by cultures growing on 2,4-DNT, and may increase NA potential where DNT/TNT mixtures are present. This research focused on understanding the fate and extent of TNT disappearance during DNT mineralization. Chemostat and  $^{14}\text{C}$  studies demonstrated that TNT cometabolism was initiated in the presence of DNT, but DNT cosubstrates were ineffective in promoting TNT mineralization. Additionally, TNT did not inhibit 2,4-DNT degradation noticeably, but 2,6-DNT degradation activity was sensitive to the presence of TNT.

The final studies were conducted to assess leaching and fate in TNT contaminated soils under conditions used in the DNT BioPilot. The leaching concentration of TNT was far lower than expected and did not explain losses in the soil. This suggests that additional processes contributed to TNT removal in pilot systems. To investigate the fate of TNT metabolism,  $^{14}\text{C}$  tracer studies were conducted in microcosms established with pilot effluents. TNT was transformed through oxidative attack, but that the extent of mineralization was very low as measured by  $^{14}\text{CO}_2$ . These observations suggest that leaching and oxidative metabolic processes caused removal of TNT in historically contaminated soils.



# CHAPTER 1

## INTRODUCTION

Isomers of dinitrotoluene (DNT) are particularly common nitroaromatic pollutants and the remediation of DNTs has drawn considerable attention for almost two decades (1). Considerable information exists on the biodegradation of nitroaromatics in pure and mixed cultures (1-4) and it is possible to identify site-specific conditions responsible for the persistence and mobility of the nitroaromatics, such as the absence of oxygen or other nutrients (5). Research has been conducted on the metabolism of DNTs under both anaerobic and aerobic conditions (1, 3, 6). Under anaerobic conditions DNTs are transformed or cometabolized, which result in incomplete destruction and difficult control (7-9). Under aerobic conditions DNTs depletion and oxygen consumption are coupled with proton and nitrite evolution, CO<sub>2</sub> production, and biomass growth (10-12). Studies under aerobic conditions have focused on pathways of DNT degradation, and ex-situ bioremediation as a treatment option at contaminated sites (13-16).

Because DNT is a microbial growth substrate and many biologically based ex-situ solutions for DNTs have proven useful in achieving desired remediation goals, DNT may be a candidate for *in situ* bioremediation, and perhaps natural attenuation (NA). A literature review that summarizes existing information on DNT biodegradation and applications of monitored natural attenuation is presented as Chapter 2 of this thesis. NA refers to the reduction of toxicity and concentration of contaminants in soil or groundwater by physical, chemical, or biological processes with minimal human intervention. NA offers ecological improvement, simplicity in concept, aesthetic advantages and increasing public acceptance (17). To date, the effectiveness of NA to

manage DNT contaminants has not been rigorously evaluated, presumably due to the observation of recalcitrance at many sites and a lack of understanding regarding factors that limit *in situ* biodegradation (1).

Many munitions facilities are contaminated with TNT as well as DNT isomers. TNT biotransformation under aerobic and anaerobic conditions has been shown in previous studies (18-22). However, the environmental fate of TNT is poorly understood and naturally occurring microbial populations have not been identified that can utilize TNT as a primary carbon and energy source. TNT can be cometabolized while DNT is degraded by bacteria growing on 2,4-DNT (1) and bacteria capable of growth on DNT can carry out the oxidation of TNT through TNT derived catechol under aerobic condition (23). Thus *in situ* biodegradation of TNT is possible and NA is an interesting possibility at sites where DNT and TNT mixtures are present.

The overarching objective of this research was to extend previous studies of aerobic DNT and TNT biodegradation to the evaluation of biodegradation for *in situ* bioremediation applications including NA. Reported herein is research conducted in six focus areas: 1) simulated *in situ* DNT bioremediation in soil columns with historically contaminated soil; 2) determination of the bioavailability of contaminant(s), metabolite (by-products) inhibition and other potential factors (*e.g.*, dissolution of contaminants and oxygen availability) that influence assimilative capacity of DNT leaching into vadose zones; 3) employment of pilot scale systems and historically contaminated soils to assess *in situ* DNT bioremediation using water infiltration; 4) determination of the limits of DNT biodegradation processes to reduce aqueous DNT concentrations thus establishing thresholds for NA in ground water; 5) establishment of conditions where TNT

disappearance can be attributed to co-metabolism during DNT biodegradation; 6) construction and operation of a pilot scale system to assess leaching of TNT from historically contaminated soils during water infiltration and oxidative biodegradation. Results of each focus area are presented in Chapters 3–8 respectively.

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## CHAPTER 2

### BACKGROUND AND LITERATURE REVIEW

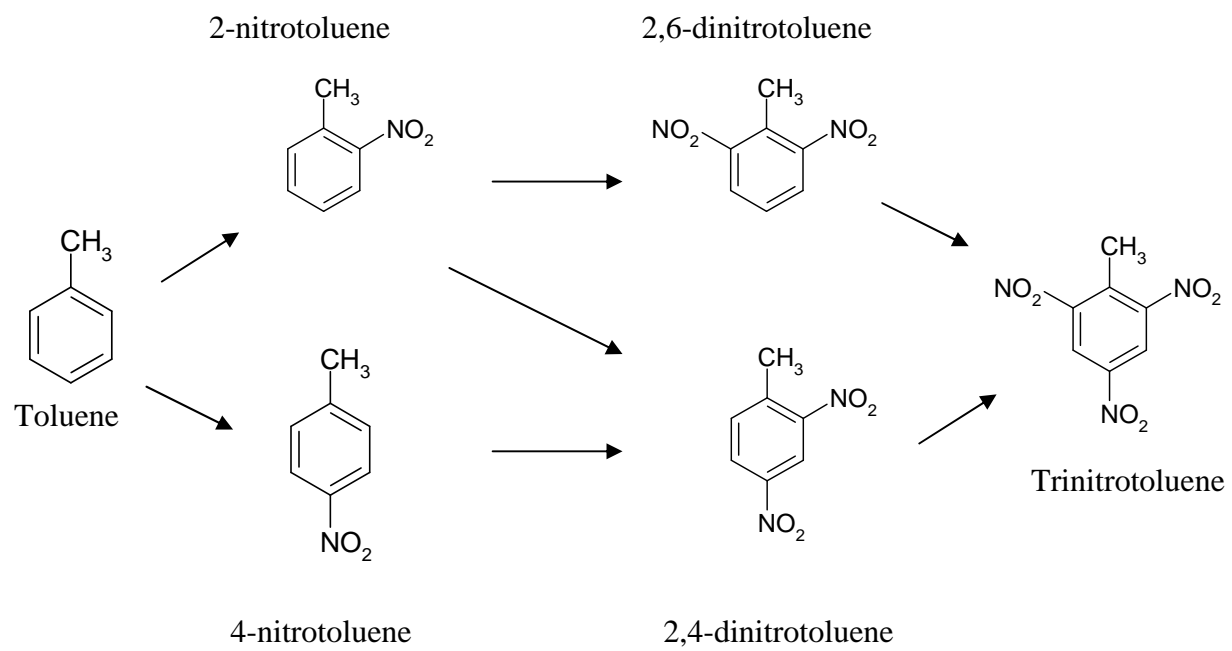
Nitroaromatic compounds are common environmental pollutants at military facilities, industrial manufacturing sites, and in mining areas. Soil and groundwater have been contaminated with these compounds as a result of previous improper disposal practices, accidental spills, and extensive use as feedstocks in industry (1-3). Many contaminated sites are not accessible to the public (1), but pressures including base closure and off-site migration in groundwater increase needs for remedial activities. Volunteer Army Ammunition Plant (VAAP) in TN is an example of a military installation, that has been extensively contaminated with nitroaromatic compounds and has initiated remediation of contaminated soil and groundwater (4-6).

In the U.S., remediation of soils that are highly contaminated with explosive compounds has been mainly conducted by excavation followed by incineration, composting, or ex-situ slurry reactors (4-9). Groundwater contaminated with nitroaromatics is usually treated by pump and treat along with activated carbon adsorption (10). More recently, *in situ* soil and groundwater bioremediation has been attempted, in an attempt to reduce the costs associated with the remediation (10, 11). Current research and technology development support lower cost alternatives such as bioremediation, in particular the development of biodegradation strategies for nitroaromatics and their precursors, or synthesis bi-products that can be used as carbon and energy sources for microorganisms (3).

This chapter is organized into four sections. The first section describes the formation of, and chemical characteristics of certain nitroaromatic compounds, specifically two dinitrotoluene isomers (DNTs) and 2,4,6-trinitrotoluene (TNT), which are commonly found at military and industrial sites. The second section focuses on the current knowledge on nitroaromatic biodegradation. The third section focuses on nitroaromatic remediation strategies, followed by a fourth section in which a common problem associated with nitroaromatic biodegradation (*i.e.*  $\text{NO}_2^-/\text{NO}_3^-$  formation) is addressed.

## 2.1. Nitroaromatic Pollutants

Nitroaromatics are aromatic organic compounds that contain one or more nitro ( $\text{NO}_2^-$ ) group(s) and form an important group of recalcitrant xenobiotic pollutants. Nitroaromatic compounds are prevalent at military sites, with TNT and DNT isomers as the most commonly found chemicals within this class (nitrobenzene, nitroxylenes, and nitronaphthalenes are less common) (3). TNT is one of the most common bulk nitroaromatics and has been used as an explosive in military munitions and in civilian mining and quarrying activities for past 100 years or more (3, 12). Even though it has not been produced in the U.S. for some time, TNT and related nitroaromatic compounds are still the most common explosive components found in soil and groundwater. TNT synthesis is conducted through sequential nitrations of toluene as shown in Figure 2.1. The first nitration produces 2-nitrotoluene and 4-nitrotoluene. The next nitration steps yield 2,4-DNT and 2,6-DNT in approximate 4:1 ratio, followed by TNT production (13). In most large scale manufacturing sites, each nitration step is conducted in nitrators



**Figure 2.1.** TNT production by a three step nitration of toluene (13).



that are physically separated for safety reasons. Thus at different locations within a facility there will be varying primary contaminants depending on the location of the different synthesis steps taking place in that area. For example, 2,4-DNT and 2,6-DNT are often found together as primary contaminants near dinitrators, but can also be found with TNT as they are principal impurities during TNT production (14). DNTs are also an important industrial chemicals used as the precursor of toluene diisocyanate (TDI) for the manufacture of polyurethane foams (15). Due to their toxicological properties, both 2,4-DNT and 2,6-DNT are classified as a priority pollutants by U.S. Environmental Protection Agency (US EPA) (13, 16, 17). EPA discharge limits in water are 0.32 mg/L for 2,4-DNT and 0.55 mg/L for 2,6-DNT (18).

Characteristics common to DNTs and TNT include electron deficient  $\pi$ -orbitals and high redox potentials, which result from the electron withdrawing nature of aryl nitro-substituents. These characteristics become more pronounced with an increasing number of nitro-groups attached to an individual aromatic ring. The presence of nitro groups hampers electrophilic attack by oxygenase of aerobic bacteria that leads to ring fission and eventual mineralization of the aromatic ring (12, 19, 20). For this reason, metabolic attack of the nitro groups, instead of the aromatic ring, has been frequently reported during biodegradation of TNT (19). Importantly, however, oxidative mineralization of DNTs have been reported with the release of nitro-groups (21-24).

## **2.2. Metabolic Pathways of Nitroaromatics Biodegradation**

### ***2.2.1. Degradation Pathways of 2,4-dinitrotoluene and 2,6-dinitrotoluene***

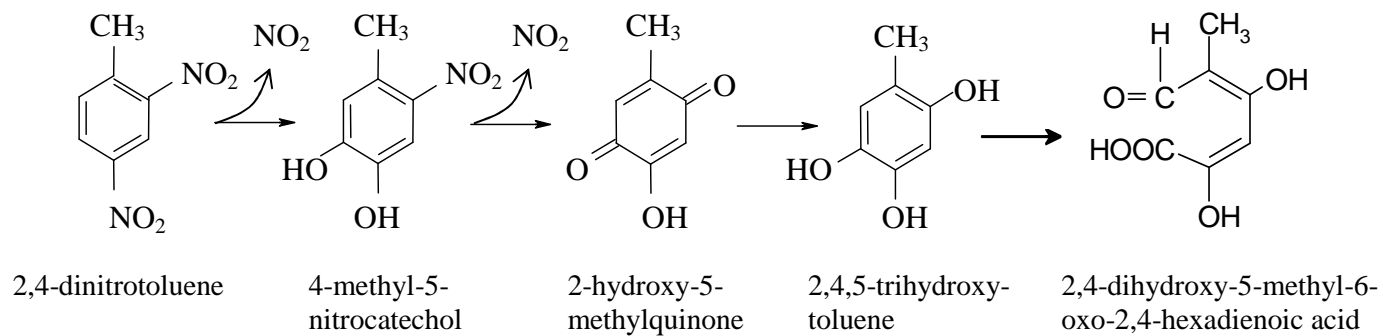
DNTs may undergo a variety of transformations under environmental conditions. One of the most commonly reported transformations has been cometabolic reduction of one or more nitro-groups (25). Cometabolism generally results in nonspecific nitro reduction that does not lead to ring cleavage. The reduced intermediates are believed to be responsible for much of the toxicity attributed to DNT (26). Because cometabolic reduction is difficult to control and does not completely destroy the contaminants, bioremediation strategies focus on the aerobic biodegradation of DNTs (10, 27). Under aerobic conditions, certain microorganisms grow on DNT isomers as sole carbon, nitrogen, and energy sources. Final products of DNT biodegradation are nitrite, carbon dioxide, water, and biomass. Bacteria capable of degrading 2,4-DNT and 2,6-DNT have been isolated and the degradation mechanisms have been described (13, 18, 28). Strains capable of growth on DNT isomers (*i.e.*, 2,4-DNT and 2,6-DNT) have been isolated from bioreactors receiving mixtures of DNT isomers (23, 28). Some of the DNT degrading strains have been identified by 16S rRNA analysis and are listed in Table 2.1 (28). The 2,4-DNT degradation pathway was first determined in *Burkholderia* sp. strain. Even though more than 30 strains that degrade 2,4-DNT have been isolated from soils and surface water, all of them appear to use the same degradation pathway as *Burkholderia* sp. strain. It has been noted that the lag phase during DNTs degradation may be strain specific (*i.e.*, JS872, PR7, and R34) (28). The bacterial degradation pathway for 2,4-DNT involves two sequential oxygenase reactions that lead to the removal of the nitro-groups (Figure 2.2a). A dioxygenase is responsible for the removal of the first nitro-substituent, by converting 2,4-DNT to 4-methyl-5-nitrocatechol yielding nitrite (24). The removal of the second

**Table 2.1.** Identification of selected DNT degrading strains (28).

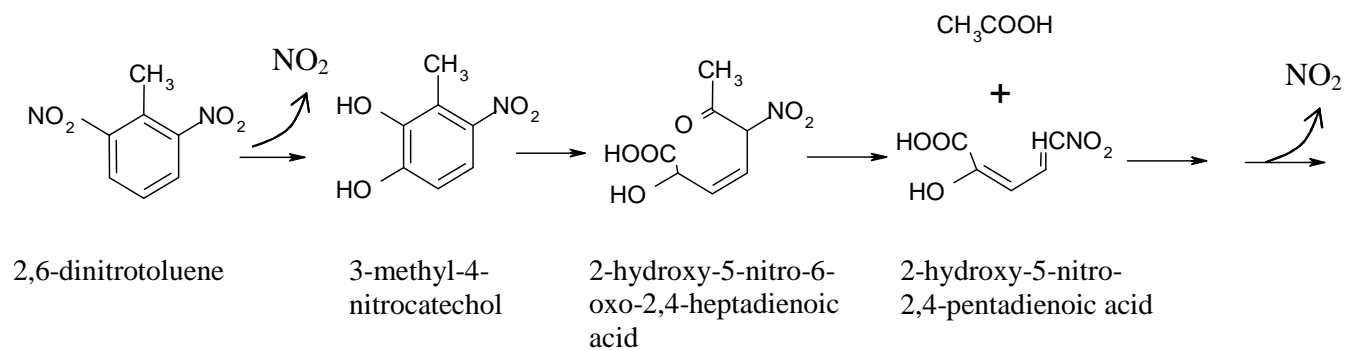
Strain	Identification	Source <sup>a</sup> ;	DNT isomer degraded
	16S rDNA	yr isolated	
R34	<i>Burkholderia cepacia</i>	1;1992	2,4
PR7	<i>Burkholderia cepacia</i>	1;1992	2,4
JS850	<i>Burkholderia cepacia</i>	3;1995	2,6
JS863	<i>Hydrogenophaga palleronii</i>	2;1995	2,6
JS867	<i>Alcaligenes</i> sp	2;1995	2,4
JS871	<i>Alcaligenes</i> sp	2;1995	2,4
JS872	<i>Burkholderia cepacia</i>	2;1995	2,4
JS881	<i>Psudeomonas putida</i>	1;1992	2,6
JS992	<i>Burkholderia cepacia</i>	2;1995	2,4 and 2,6

<sup>a</sup> Source: 1, Radford Army Ammunition Plant, soil and surface water; 2, Volunteer Army Ammunition Plant, soil and surface water; 3, West Virginia, activated sludge

(a)



(b)



**Figure 2.2.** Degradation pathways for (a) 2,4-dinitrotoluene and (b) 2,6-dinitrotoluene (13).

nitro-substituent is achieved by a monooxygenase (MNC monooxygenase), which converts the 4-methyl-5-nitrocatechol into 2-hydroxy-5-methylquinone (29). The resulting methylhydroxyquinone is later reduced to 2,4,5-trihydroxytoluene, which is then transformed to 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid (meta cleavage), in a reaction catalyzed by an extradiol dioxygenase (21). Finally these intermediates are completely oxidized to carbon dioxide through the Krebs cycle (14).

The 2,6-DNT degradation pathway is similar to that of 2,4-DNT degradation except for the initial steps (Figure 2.2b). Both 2,4-DNT and 2,6-DNT are initially transformed by a dioxygenase attack; in the case of 2,6-DNT, the reaction yields 3-methyl-4-nitrocatechol (3M4NC) with the removal of ortho-nitro group. The 3M4NC ring is opened by a catechol-2,3-dioxygenase resulting in 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid (HNOHA). HNOHA is later converted to 2-hydroxy-5-nitropenta-2,4-dienoic acid (HNPA) in a reaction that is catalyzed by an partially purified hydrolase (14, 20). The remaining nitro group is eliminated in subsequent uncharacterized reactions (20, 28).

2,4-DNT degradation capabilities of most 2,4-DNT degraders are not affected by relatively high concentrations of 2,4-DNT or the presence of 2,6-DNT (28). However, 2,6-DNT degradation has been shown to be strongly inhibited by the presence of elevated concentrations of either of DNTs (13). Spain *et al.* have reported inhibition of inducing the 2,6-DNT degradation pathway at 2,6-DNT concentration above 100  $\mu\text{M}$  while growth of fully induced cells on 2,6-DNT at 2,6-DNT concentration below 500  $\mu\text{M}$  (13). There is evidence of inhibition of 2,6-DNT degradation in the presence of 2,4-DNT. 2,6-DNT degradation was inhibited by 2,4-DNT concentration of 500 and 1,000  $\mu\text{M}$ , and higher

concentration of 2,4-DNT (2,500  $\mu\text{M}$ ) irreversibly limited 2,6-DNT degradation (6). Unfortunately, at most sites contaminated with munitions the ratio of 2,4-DNT to 2,6-DNT is likely to be approximately 4:1, which means they usually coexist in the environment, resulting in potential inhibitory effects. Even though bacteria which can degrade DNTs are present at contaminated sites, the 2,6-DNT may not be removed effectively by biological systems, due to this reported inhibition. Therefore, inhibition impacts on the biodegradability of individual DNT isomers caused by coexistence represent a significant concern in the design and operation of remediation strategies.

### ***2.2.2. Biodegradation of 2,4,6-Trinitrotoluene***

Concerns regarding the recalcitrance and fate in the environment of TNT have resulted in a number of research studies that investigated biological degradation and treatment strategies for TNT contamination in soil and groundwater (10, 20, 30). Studies have demonstrated that under aerobic and anaerobic conditions, TNT can be biotransformed including the sequential reduction of the aryl nitro-groups to the hydroxylamine or amine level, generally with poor mass balances (31, 32). The primary reason for the poor mass balances is attributed to the further conversion to soil bound residues under anaerobic conditions. Using  $^{14}\text{C}$ -TNT, it was shown that soil bound residues account for 30 % to 70 % of the initial radioactivity (33).

Amino-dinitrotoluenes (ADNTs) and diaminonitrotoluenes (DANTs) produced by reduction of the nitro groups of TNT are commonly cited metabolic products of TNT and the product of complete TNT reduction under strong reducing conditions is 2,4,6-triaminotoluene (TAT) (31, 32, 34-37). TAT has been postulated as a central

intermediate in the anaerobic treatment of TNT (38), even when it was not detected. The amino groups of TAT are strong ring activators, making the ring susceptible to electrophilic attack (12, 39). Additional products from condensation, ring reduction and ring substitution have been identified (12, 36, 40). The oxidation of TAT can be catalyzed in the absence of cell by trace elements such as  $Mn^{2+}$  (19), but the products of this process appear to be polymers that are at best difficult for bacteria to degrade. In anaerobic systems TAT may react to form tetra-amino-azobenzenes or polynuclear azo compounds (36, 41-43).

The final products of TNT biodegradation are best described as either reversibly bound to soil or undefined polar metabolites that remain in the aqueous phase. Thus it does not appear that the anaerobic reduction of TNT results in the formation of ring fission precursors that are amenable to complete destruction. Humification has been proposed as an end-point for treatment of excavated residues in anaerobic reactors where external carbon sources can be added with maintenance of low redox potentials (7, 8).

Despite several reports regarding TNT serving as a nitrogen source in biological systems, no studies have shown that microorganisms can grow on TNT as a primary energy or carbon source (9, 14, 44, 45). Genetically modified bacteria have been developed to use TNT not only as nitrogen source (46, 47) but also as a final electron acceptor in respiratory chain (48) under anoxic conditions. These findings provide the possibility of remedial applications in anaerobic conditions contaminated with TNT.

The degradation of TNT by fungal species have been studied in depth, and in most cases the amount of mineralization was higher than those obtained by bacteria (49-53). The ligninolytic (nitrogen-limiting) or nonligninolytic (nitrogen-sufficient) conditions used in

the culture medium determined the extent of TNT mineralization (49). It was observed that white-rot fungi perform cometabolic TNT mineralization through an oxygenolytic degradation and fungi-mediated radical attack on the aromatic ring, which subsequent reduction of one or more nitro-groups (50, 54).

A novel pathway for TNT metabolism was recently investigated through  $^{14}\text{C}$ -TNT studies under aerobic conditions. This study demonstrated an oxidative microbial degradation of TNT via a novel oxidative metabolite of TNT (*i.e.*, 3-methyl-4,6-dinitrocatechol) (55). Other evidence for oxidative attack on TNT appeared during the operation of the pilot scale system at Volunteer Army Ammunition Plant (VAAP) (14). Mixture of 2,4-DNT and TNT were continuously applied to the system. The results presented that TNT disappeared from the system when 2,4-DNT was being degraded, but TNT was not degraded in the absence of 2,4-DNT. This observation suggests that this mixture of contaminate may serve to increase the potential for treatment of TNT which is considered as a recalcitrant compound at many sites.

Because TNT and DNT are often found as co-contaminants and because TNT metabolites may share DNTs degradation pathways, some form of TNT cometabolic biotransformation is possible in combination with DNT degradation, and may partially support microbial growth. The development of *in situ* DNT bioremediation system is ongoing, and the extension of developments in technology may be extended to TNT remediation, if researchers dismiss the belief that TNT is non-biodegradable. If achieved, many opportunities to apply bioremediation at abandoned contaminated sites will exist.

### ***2.2.3. Stoichiometry for Growth on DNT***



Zhang and Daprato (4, 5) used thermodynamics to predict the stoichiometry of DNT degradation based on a method described by Rittmann and McCarty (56). In this analysis, bacterial energetics involves two components, one for cellular synthesis and the other for energy. When microorganisms use a substrate for the production of cell material, a portion of the energy flow is transferred to a terminal electron acceptor to generate energy, the other portion of the energy released is stored in cellular materials. In order to determine the overall reaction (R) for biological growth, the energy reaction and the synthesis reaction are developed with half-reactions for the electron acceptor ( $R_{ea}$ ), the electron donor ( $R_{ed}$ ), and cell material ( $R_c$ ) presented below (56, 57).

$$R = R_{ed} - f_e R_{ea} - f_s R_c \quad (2.1)$$

where

$$f_e + f_s = 1.0 \quad (2.2)$$

The half-reactions are written on an electron-equivalent basis, and the fractions of electrons used for energy generation ( $f_e$ ) and cellular synthesis ( $f_s$ ) must sum to 1.0. Therefore, the values of  $f_s$  and  $f_e$  must be known to determine the overall balanced stoichiometric equation for the biodegradation of DNT. These values can be calculated from the value of A.

$$f_s = \frac{1}{1 + A} \text{ or } f_e = 1 - f_s = \frac{A}{1 + A} : \quad ** \text{ Decay ignored} \quad (2.3)$$

The term A (equivalent electron donor converted to energy/equivalent cells synthesized) can be calculated from an energy balance. Bioenergetics based on thermodynamic principles (*i.e.*, free energy changes) govern the overall stoichiometry of substrate utilization and microbial growth. The energy released by the oxidation is A( $\Delta G_r$ ), where  $\Delta G_r$  is the free energy released per equivalent of electron donor oxidized for energy generation. An energy balance must be maintained around the energy carrier at steady state:

$$A(\Delta G_r) - (1 - \epsilon)A(\Delta G_r) = \Delta G_s \quad (2.4)$$

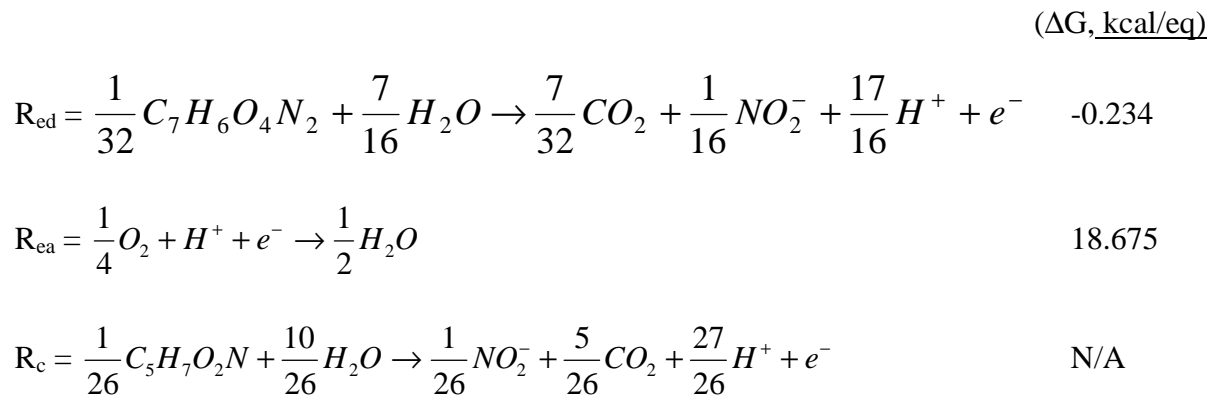
$$A = \frac{\Delta G_s}{\epsilon \Delta G_r} \quad (2.5)$$

where

$$\Delta G_r = \frac{\text{kcal energy available}}{\text{equivalent } e^- \text{ donor used for energy}}$$

$$\Delta G_s = \frac{\text{kcal energy required}}{\text{equivalent cells synthesized}}$$

To calculate  $\Delta G_r$  and  $\Delta G_s$  values, the half reactions for oxidation of DNT shown below are required:



The  $\Delta G$  value for the DNT reaction presented above was calculated by Daprato (4) using the group contribution method developed by Mavrovouniotis (58) with the  $\text{NO}_2^-$  contribution determined by Shelly *et.al.* (59). The standard free energy ( $G_f^\circ$ ) of formation of the DNT was used to calculate the  $\Delta G$ . Equation 2.6 is used to predict the  $G_f^\circ$  for a compound using an estimation technique based on the molecular structure of DNT (60).

The free energy of formation values published by Mavrovouniotis, and Brock and Madigan (58, 61) were used to calculate the standard free energy of formation of the DNT half reaction. The contributions for the DNT groups are estimated (Table 2.2).

$$G_{f298}^\circ = 53.88 + \sum_{i=1}^n N_i \Delta G_i \quad (2.6)$$

The  $G_f^\circ$  of the overall stoichiometric components for the determination of  $\Delta G$  are listed in Table 2.3.  $\Delta G_r$  calculated from  $R_{ea}$  and  $R_{ed}$  is  $-18.909$  kcal/equiv.  $\Delta G_s$  is the energy required to synthesize one equivalent of cells from a given carbon source, which is made up of components: 1)  $\Delta G_c$ , 2)  $\Delta G_n$ , and 3)  $\Delta G_p$ .  $\Delta G_c$  is the free energy required to convert pyruvate carbon to cellular carbon, which is  $7.5$  kcal/equiv when ammonia is used as cellular material. In this case, the nitrogen source is  $\text{NO}_2^-$  so the free energy required to convert the nitrogen source to  $\text{NH}_3$  ( $\Delta G_n$ ) is calculated, where the value of  $\Delta G_n$  is  $3.25$  kcal/equiv.  $\Delta G_p$  is the free energy required to convert the carbon source to pyruvate ( $=\Delta G_{\text{pyruvate}} - \Delta G_{ed}$ ).  $\Delta G_p$  for heterotrophic case is taken at  $8.31$  kcal/equiv.

**Table 2.2.** Atomic group contributions to estimate  $\Delta G_{f298}$  for DNT.

Atomic Group	$\Delta G$ (kcal/mol)
=CH (aromatic)	8.6
=C (aromatic)	1.1
-NO <sub>2</sub>	6.9
-CH <sub>3</sub>	8.5

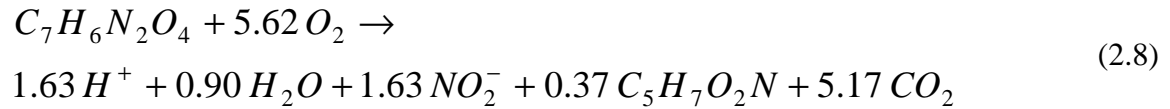
**Table 2.3.** Free energies of formation of DNT  $G_f^0$ .

Compound	$G_f^0$ (kcal/mol)
CO <sub>2</sub>	-94.32
H <sub>2</sub> O	-56.60
H <sup>+</sup>	-9.5
NO <sub>2</sub> <sup>-</sup>	-8.91
C <sub>7</sub> H <sub>6</sub> N <sub>2</sub> O <sub>4</sub>	30.90

Finally, when this energy is transferred to the energy carrier, a portion again is lost through transfer inefficiencies, so it is presumed 60 % efficient (56), which is described below by  $\epsilon$ .

$$\Delta G_s = \frac{\Delta G_p}{\epsilon} + 7.5 + \frac{\Delta G_n}{\epsilon} = 26.8 \text{ kcal/equiv} \quad (2.7)$$

Based on “A” calculated using  $\Delta G_s$  and  $\Delta G_r$  and Equation 2.6,  $f_s$  and  $f_e$  are 0.30 and 0.70 respectively. These values were applied with the half reactions shown to Equation 2.1 to write an overall stoichiometric equation of DNT oxidation.



The stoichiometric coefficients can be used to predict the rate and molar ratio of other products and reactants in Equation 2.8. Production of nitrite is generally taken as an indicator of DNT biodegradation.

## **2.3. *In Situ* Bioremediation and Natural Attenuation (NA) of DNT**

### **2.3.1. *In Situ* Bioremediation**

*In situ* bioremediation takes advantage of biological processes to destroy harmful chemicals in the environment (*e.g.*, soil and surface or subsurface water with minimal disruption). During bioremediation, microbiological processes degrade or transform

contaminants to less toxic or harmless forms such as carbon dioxide and water (62, 63). For microorganisms to grow and survive, nutrients, carbon, and appropriate environmental conditions (*e.g.*, temperature and oxygen availability) must be available.

NA, which is defined as the decrease in contaminant concentrations due to dispersion, dilution, sorption, volatilization, and biodegradation is most successful when biodegradation processes are a dominant process for the destruction of contaminants (64). Interest in NA of environmental contaminants has increased in recent years as the result of the inherent costs associated with more invasive remedial technologies, such as pump-and-treat systems, which were common remediation technologies in the past and are still being used. The US EPA recommends that field or microcosm studies are conducted with contaminated site media to provide a line of evidence that destruction of contaminants is actively occurring through biodegradation (65). If biological degradation is observed through these tests, results obtained can be used as a base line for monitoring natural attenuation (MNA) on site(s).

If the nutritional and physiological requirements, varying from site to site, are not adequate, bioremediation may be limited. Thus alternative strategies have been used that include various practical methods for implementing enhanced *in situ* bioremediation through application of nutrients or electron acceptors/donors (referred to as *biostimulation*). As an example, oxygen concentration might be increased by delivering chemical additives that evolve O<sub>2</sub> (14, 63). Oxygen is an electron acceptor used by organisms capable of rapid aromatic biodegradation (14). In some cases, microorganisms capable of degrading target compounds are not present in the soil, and therefore have to be introduced to increase the rate or extent of pollutant degradation (referred to as

*bioaugmentation*). By definition, such engineered solutions would not be NA, but still fall within the realm of *in situ* remediation systems.

### **2.3.2. Natural Attenuation of DNT**

DNTs are biodegradable compounds that are good candidates for NA including biodegradation. In some instances, considerable information exists on the DNT's biodegradation mechanisms in pure cultures and successful remediation technology has been reported for site clean-up (6, 10, 14, 22, 23, 66). Additionally, possible identification of site-specific conditions responsible for the recalcitrance and degradation of DNTs (14, 67) and the discovery of bacteria that are able to degrade DNT compounds at DNT contaminated sites improve DNTs potential for remediation by NA. If biodegradation processes can mainly control or destroy DNTs, NA may work well to minimize risks associated with contamination with DNTs. Other cases, however, are not simple because assessment of DNT's fate in the environment is complex and little is known about factors that influence *in situ* DNT remediation including natural attenuation.

The cases examples of sites at which NA has been carefully studied were described by several researchers. The potential for DNT NA was studied at site where groundwater was contaminated with DNT at an army ammunition plants (14). Naturally occurring aerobic bacteria capable of metabolizing DNT have been identified at Badger Army Ammunition Plant (BAAP), WI (4, 67-69). Previous work on soils from the Badger's Propellant Burning Ground demonstrated that rapid degradation of DNT can occur in the presence of indigenous bacteria as long as sufficient nutrients were present (13). In microcosm studies, vadose zone DNT samples taken from the contaminated site

were degraded over a 7 day incubation without nutrient addition (69). 2,4-DNT degrading bacteria was isolated, and microcosms containing DNT contaminated soils demonstrated 2,4-DNT degradation along with stoichiometric release of nitrite (6, 13). Field studies also demonstrated that destruction of DNTs in the groundwater plume was attributed to biological activity (13). Combined, these observations suggested that biodegradation processes are taking place at that location without any intervention.

In case of Weldon Spring Ordnance Works (WSOW) in MO which has a DNT plume as a result of manufacture of explosives during World War II (1, 70), monitoring data showed a decrease of 2,4-DNT and 2,6-DNT in the flow path of groundwater and led to *in situ* microbial studies. Microcosms were constructed with contaminated soils and radiolabeled-DNTs (2,4-DNT and 2,6-DNT) to demonstrate DNT NA. The significant mineralization of 2,4-DNT was observed as measured by  $^{14}\text{CO}_2$ , while 2,6-DNT mineralization was very low. Product characterization included mineralized forms, ADNTs as reduced forms, and other unidentified products. In some cases, however, the DNT remained unchanged. It was also reported that the toxic characteristics of reduced metabolites, which did not bind to soil, made the problem even worse (71). Based on these results, DNT NA may not be appropriate for the Weldon Spring site, as the incomplete transformation or sequestration does not lead to complete mineralization or the production of less harmful compounds.

#### **2.4. Removal of Nitrite and Nitrate**

As was previously mentioned, biological degradation of DNT and TNT results in the release of large quantities of nitrite. In many cases this nitrite release can be used as

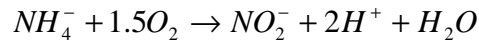


an indicator of DNT degradation (21-24, 28, 67). Nitrate is sometimes found at contaminated sites. Since nitrite and nitrate are highly soluble in water and have low tendency to sorb to soils, they have a high potential to migrate to groundwater. Due to the high migration potential and the toxicity of nitrite/nitrate to living organisms, it is necessary that low concentrations are maintained in waters to complete remediation. This section describes biological mechanisms that can be used to remove nitrite/nitrate from water.

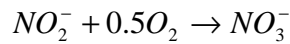
#### **2.4.1. Biological Nitrification**

Nitrifying bacteria are autotrophic bacteria capable of obtaining energy from the oxidation of ammonia or nitrite (57, 63). They are obligate aerobes, and capable of growing with oxygen as electron acceptor and with a combination of CO<sub>2</sub> and organic compounds as carbon source. Compared to purely autotrophic growth, the addition of organic compounds stimulates cell growth and increases cell yield (72). In heterotrophic nitrification, organic and inorganic nitrogen compounds are oxidized when an external energy source is available (73). However, it is doubtful that heterotrophic organisms cause significant production of nitrate, since they have a slower rate than autotrophic organisms (57). The heterotrophic nitrification may be more important in atypical environments (*i.e.*, extremely high alkaline or acidic conditions) (57). The nitrification in soil or waste water treatment processes is attributed primarily to *Nitrosomonas* and *Nitrobacter*, which oxidize ammonia to nitrite and then to nitrate, respectively (57, 63). Reactions representing the general two-step oxidation (ammonia to nitrate) process for energy-generation is shown in the following equations:

*Nitrosomonas*



*Nitrobacter*



Combining these two steps gives the final stoichiometry

Total Reaction



For the previous reactions, complete oxidation of ammonia to nitrate requires 4.57 g of O<sub>2</sub> per g of N consumed with 3.43 g/g used for ammonia oxidation and 1.14 g/g used for nitrite oxidation. When cellular synthesis is considered, less oxygen is required. The actual oxygen consumption is expected to be close to 4.20 – 4.33 g O<sub>2</sub>/g N consumed accounting for both cellular synthesis and sequential oxidation (74).

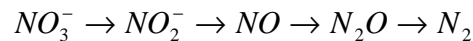
Nitrifying organisms are known to be susceptible to toxicity or inhibition (57).

The toxicity may be sufficient to stop nitrification activity temporarily or kill the nitrifying bacteria unless it is removed or diluted and new growth occurs. Some inhibition factors include temperature, pH, dissolved oxygen (DO) concentration, organic loading, and the amount on nitrite and nitrate (75). Reports on the effect of temperature and pH on nitrification rates vary in the literature, but nitrifying bacteria show optimal activities at mesophilic temperatures and neutral to alkaline pH values (6.5–8.5) (63). At lower temperatures nitrifying bacteria are more significantly influenced by varying pH. The reported optimal DO concentration varies widely for different experimental

conditions that used either enriched cultures in laboratory tests or activated sludge systems for wastewater treatment. It has been reported that a minimum DO concentration needed for nitrification is 0.3 mg/L. The growth rate according to DO change between 1.0 mg/L and saturation concentration was affected by temperature (74). In addition, there is a report on organic compound toxicity (76), and it is known that the concentrations higher than 0.2 – 2.8 mg HNO<sub>2</sub>/L inhibit nitrite oxidizing bacteria (77), and the produced nitrate is inhibitory at concentrations between 30 and 65 mM (78).

#### **2.4.2. Biological Denitrification**

Denitrification or dissimilative nitrate reduction is coupled to respiration and proceeds the sequential reduction of nitrate to nitrite, nitric oxide, nitrous oxide, and N<sub>2</sub> gas as shown below (57, 79).

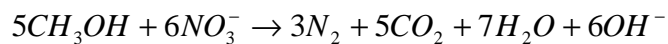


The denitrifiers are chemotrophs and facultative aerobes, which means that they require an organic or inorganic electron donor and they can use oxygen as well as nitrate or nitrite (79). When oxygen is present, the microorganisms use oxygen instead of nitrate by providing a better electron acceptor for metabolism (79). Oxygen can control denitrification in two ways. The first is the effect of oxygen on the initiation of denitrification. McKenney examined kinetics of denitrification with varying oxygen concentrations, which indicate that concentrations greater than a few tenths of percent of O<sub>2</sub> in oxygen and nitrogen mixture inhibit the activity of denitrifying bacteria (80, 81).

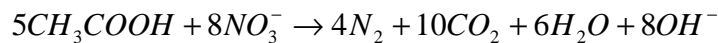
The second control mechanism is repression of denitrification rate by oxygen concentration. The denitrification rate at a 0.20 mg/L of dissolved oxygen (DO) concentration was lower about one-half than at a zero DO concentration. When the DO concentration was 2.0 mg/L, the denitrification rate decreased to 10 % of the zero DO concentration (74). In contrast of inhibitory effect of DO, the effect of pH on denitrification has been less concern due to production of alkalinity during denitification reactions (74).

Major factors affecting denitrification also include the amount of substrate (*i.e.*, electron donor) necessary to oxidize nitrate or nitrite. Easily degraded substrates such as methanol and acetic acid cause the highest rates of denitrification. The following equations show redox reactions proposed for various substrates used for denitrification (79).

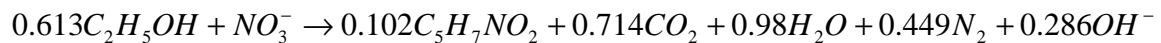
Methanol (74)



Acetic Acid (79)



Ethanol (82)



The influent chemical oxygen demand (COD) to nitrogen ratio (COD/N ratio) is one of most important factors on the denitrification processes (83) which is commonly

considered for the design of denitrification reactors (74). Methanol is the most widely studied and most commonly preferred substrate on a low sludge yield and cost when an external carbon source is required. Based on the above formulation, the methanol (as COD) required to nitrate nitrogen ratio is 3.71. In case of the reaction with ethanol stoichiometric COD/N ratio was 4.2 (83). The above formulation also presents that denitrification processes produce alkalinity ( $\text{OH}^-$ ) and thus the pH is usually elevated in contrast to nitrification processes. Although the effect of pH on the denitrification rates is of less concern, a pH outside the optimal range might affect bacterial metabolism or can lead to accumulation of intermediates (*e.g.*, nitrite accumulation) (84). The optimal pH is ranged form 7 to 8 (82, 85, 86).

Denitrification is widespread among heterotrophic and autotrophic bacteria, many of which can shift between oxygen respiration and nitrogen respiration. The following genera are listed as the heterotrophic organisms (74): *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Chromobacterim*, *Corynebacterium*, *Flavobacterium*, *Hypomicrobium*, *Moraxella*, *Neisseria*, *Paracoccus*, *Propionibacterium*, *Pseudomonas*, *Rhizobium*, *Rhodopseudomonas*, *Spirillum*, and *Vibrio*. In addition, archaea such as *Halobacterium* and *Methanomonas* can denitrify (74). Autotrophic organisms use carbon dioxide or bicarbonate as a carbon source instead of organic carbon and use hydrogen and reduced sulfur compounds as electron donors (*e.g.*, *Paracoccus* denitrifiers and *Thiobacillus denitrificans*) (74). Because of their great metabolic diversity, denitrifying bacteria are ubiquitous in soils, sediments, surface water, groundwater, and wastewater treatment plants.

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## CHAPTER 3

### ***In Situ* Bioremediation of DNT Contamination in Historically Contaminated Soils—Microcosm and Bench Scale Column Studies**

#### **3.1. Overview**

In this chapter, laboratory studies are presented that focus on the ability to stimulate DNT biodegradation in historically contaminated soils from an abandoned industrial site in Barksdale WI. More specifically research evaluated factors controlling, and the potential for *in situ* bioremediation of DNT as a possible remediation strategy, using a matrix of shake flask (microcosm) and bench scale soil column experiments. Degradation rate and extent of 2,4-DNT varied in initial shake flask experiments among the various sample taken at different location at the Barksdale site. Subsequent column studies were conducted in several bench scale experiments with soils containing 2,4-DNT as the predominant contaminant. Indigenous cultures capable of mineralizing 2,4-DNT were present in these soils as demonstrated by column effluent and effluent seeded batch studies. Limited 2,6-DNT degradation was observed in the presence of 2,4-DNT. Nitrite and nitrate were observed at high concentrations, but at varying ratios, as a result of rapid DNT mineralization. Mineral media addition did not significantly enhance degradation activity. Enhanced aeration slightly increased degradation activity when 2,4-DNT is present. Enhanced buffer strength (capacity) increased and sustained biodegradation, while low pH ( $< 6.0$ ) and elevated nitrite concentrations ( $\geq 5$  mM) inhibited degradation activity.

### 3.2. Introduction

Because DNTs are relatively insoluble and do not migrate rapidly in soil systems, DNT contamination at explosives facilities is primarily found in sorbed and crystalline phase. Leaching due to infiltration can carry contaminants to the underlying vadose zone and eventually to the water table. If significant degradation within soils can be stimulated, *in situ* bioremediation may be a viable remediation process for DNT sites.

Previous studies have shown that rapid degradation of 2,4-DNT can occur in areas where extremely high levels of DNT are present (1, 2). For example, soil column studies conducted at field capacity demonstrated a rapid rate of DNT degradation possible in soils when oxygen was provided in the soil gas (1). The soils used were obtained from Badger Army Ammunition Plant (BAAP). High levels of 2,4-DNT can be biodegraded by addition of complete mineral medium, while no degradation of 2,6-DNT was observed. 2,4-DNT removal (22 to 30 %) from the column systems were observed through both washout and biodegradation, but 2,6-DNT was not found to biologically degrade under any circumstances, thus the removal (82 to 91 %) resulted from only washout. Whereas nitrite was produced from all soil columns, appreciable nitrate production was not observed during the study. A concern in DNT bioremediation is by-products of DNT degradation including nitrite, a weak acid which can accumulate and create unsuitable conditions for DNT degradation associated with low pH inhibition or nitrite toxicity (1-4).

Interest in *in situ* bioremediation or NA has increased recently as a cost effective alternative to other conventional remediation (5-11) such as ex-situ slurry reactors, composting, and incineration (4, 12). The objective of the research is to assess *in situ* soil

bioremediation as an effective alternative for remediation of DNT contaminated surface soils (Barksdale (WI), APPENDIX A). Experiments were conducted with historically contaminated soils containing varying levels of DNT. Specific objectives included: 1) Confirming the presence and activity of DNT degraders in soils, 2) Determining the soil concentrations of DNT that can be reached through bioremediation, 3) Determining the soil conditions (*e.g.*, the amounts of DNT's in soils) that cause 2,6-DNT degradation, and 4) Evaluating the effect of various environmental factors, including buffer strength, media addition, oxygen concentration, and pH, on the rate of degradation processes.

### **3.3. Materials and Methods**

#### **3.3.1. Chemical Sources.**

All chemicals and reagents used were of the highest purity available commercially, purchased from the Sigma-Aldrich Company or Fisher Scientific. Organic solvents for HPLC analysis were prepared: acetonitrile (HPLC grade, 99.9 %, Fisher Scientific), trifluoroacetic anhydride (reagent grade, Fisher Scientific), and acetic acid (glacial reagent, Acros). Standard solution (Dionex) containing  $\text{NaHCO}_3$  (1.8 mM) and  $\text{Na}_2\text{CO}_3$  (1.7 mM) was used for Ion Chromatography (IC) analysis.

#### **3.3.2. Analytical Methods.**

All aqueous samples analyzed were first filtered using a PTFE filter unit (0.22  $\mu\text{m}$ , Millipore). Separation and quantification of DNT were accomplished via HPLC (Agilent 1,100 series) equipped with a diode array detector ( $\lambda = 230$  or  $246$  nm). Two HPLC methods for DNT analysis were used: 1) A standard bond  $\text{C}_{18}$  column (Zorbax®



SB-C18) used to separate the sample constituents at a flow rate of 1 mL/min using a mobile phase of water with 0.1 % acetic acid and acetonitrile (13) and 2) a Hybercarb® porous graphite column (100 x 3 mm, 5 µm, Thermo Hypersil, UK) with a mobile phase of 96 % acetonitrile (ACN) and 4 % water with 0.55 mL/L of trifluoroacetic acid at a flow rate of 0.7 mL/min (1).

Nitrite was measured colorimetrically following Standard Methods for Water and Wastewater Treatment method 4500-NO<sub>2</sub><sup>-</sup> (14). DNT solutions (960 µL) were taken from the filtered samples and then the color reagents (40 µL) were added. After 10 minutes, nitrite concentrations were measured with a spectrophotometer (Cary 50, Varian) at a wavelength of 543 nm. Nitrate was analyzed using Dionex IC equipped with Dionex AS4A-SC column and ED40 electrochemical detector. The pH was measured with a Denver Instrument pH 220.

### **3.3.3. DNT Soil Extraction Procedure.**

Each soil sample (approximately 0.05 g) and ACN (200 µL) was added to an Ultrafree-MC 0.22 µm two chamber centrifugal filter unit (Millipore) and allowed to equilibrate (10 min) (1, 2, 15). The unit was centrifuged for 5 min at 7,000 RPM, and this process was conducted twice. The washed soil sample was removed from the top of the filter and aqueous samples (600 µL) were collected from the bottom of the unit filter to be analyzed via HPLC.

### **3.3.4. Microcosm Studies: Screening for DNT Degrading Activity.**

Shake flasks were employed using the collected soil samples (approximately 5 g soil) and mineral media (100 mL) with no external DNT added (16). Soil samples were collected from three contaminated areas (Lydol Ditch, Trivolien, and Dynamite) with corresponding site locations. All shake flasks were incubated at a constant temperature of 20°C on a rotary shaker. The pH was routinely measured and manually adjusted daily to maintain a pH of  $8.0 \pm 0.5$ . Foam stoppers were used to minimize evaporation and prevent contamination. Samples (2 mL) from active reactors were transferred to soil free microcosms containing fresh media and 80 mg/L 2,4-DNT to verify the presence of 2,4-DNT degraders in the reactors. System parameters including 2,4-DNT concentration, pH, and metabolites ( $\text{NO}_2^-$ ) were monitored in all reactors. Upon the observation of DNT degradation activity, DNT concentrations and medium volume were increased.

A second set of shake flask studies were conducted with column effluent samples to verify the ability of organisms to aerobically degrade DNT in soil columns. This study was conducted using only column effluent directly transferred to serum vials or DNT amended vials, and analyzed for DNT and corresponding end products.

A third series of subsequent shake flask samples were prepared at various initial nitrite levels and pH values to investigate the inhibition effects on DNT degradation with the goal of maintaining and optimizing DNT degradation. Nitrite was added to five flasks containing mineral media (100 mL) at concentrations of 5, 10, 20, 40, and 50 mM. Another series were prepared at pH values of 4, 5, 6, 7, and 8 without addition of external nitrite. Aliquots (10 mL) taken from highly DNT contaminated column were used as inoculum. All flasks in this study were incubated at room temperature over 8 days.

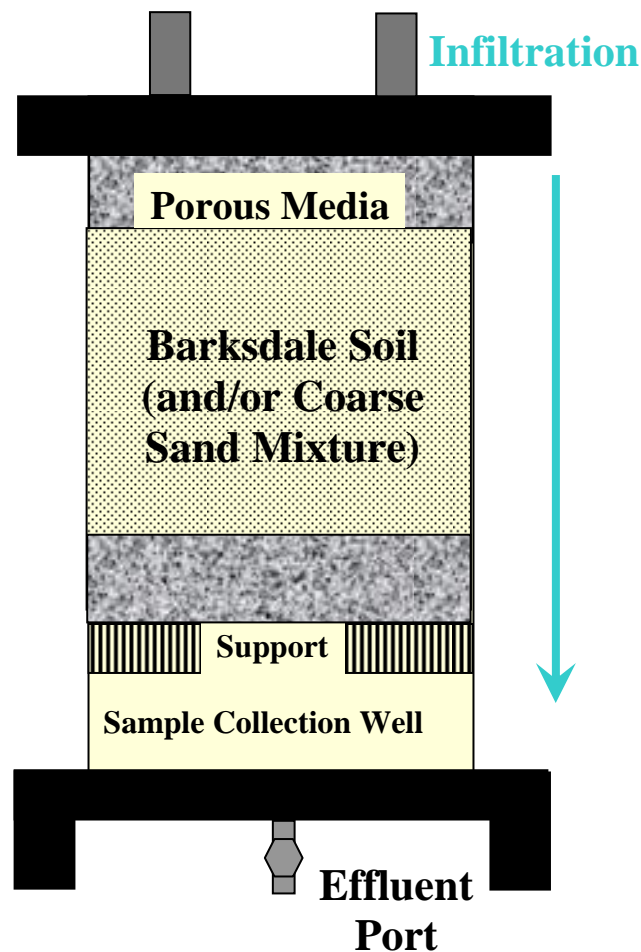
### 3.3.5. Soil Preparation for Column Studies.

Contaminated soil samples containing primary DNT isomers (*i.e.*, 2,4-DNT and 2,6-DNT) were collected from Barksdale, WI. Three types of soil were prepared and homogenized before column studies. One sample subset (highly contaminated soil with  $11,045 \pm 3414$  mg 2,4-DNT/kg soil and  $3,442 \pm 2240$  mg 2,6-DNT/kg soil) consisted of a mixture of 70 % sand (clean) and contaminated soil (*i.e.*, soil:coarse sand = 3:7 (w/w)). The other two subsets (intermediate or low DNT contaminated soils) were not diluted with sand. The DNT concentrations contained in soil matrix ranged from  $728 \pm 509$  to  $1,431 \pm 101$  mg/kg soil for 2,4-DNT and  $42 \pm 31$  to  $101 \pm 101$  mg/kg soil for 2,6-DNT.

### 3.3.6. Column Studies.

Column studies to assess DNT leaching and degradation in historically contaminated soils were conducted as described by Fortner *et al.* (1) and are depicted in Figure 3.1. Columns (10 cm internal diameter x 30 cm length) were constructed (from bottom to top) as follows: Wire mesh cloth (0.145 cm) was placed at the base of the column; a layer of gravel (3.5 cm) was placed on the wire mesh to support the soil and to prevent washout and clogging of the effluent line; contaminated soil; a layer of gravel (7.5 cm) was placed on top of the soil bed to uniformly distribute the influent. Feed solutions (100 ml) were poured into the column once per day. Column effluent was collected daily thorough a ball valve placed at the bottom of the column and analyzed for DNT, nitrite, nitrate, and pH. Each column was named according to the extent of background nitroaromatic (DNT) concentrations: Highly Contaminated Soil (**HS**) Column (1EA), Medium Contaminated

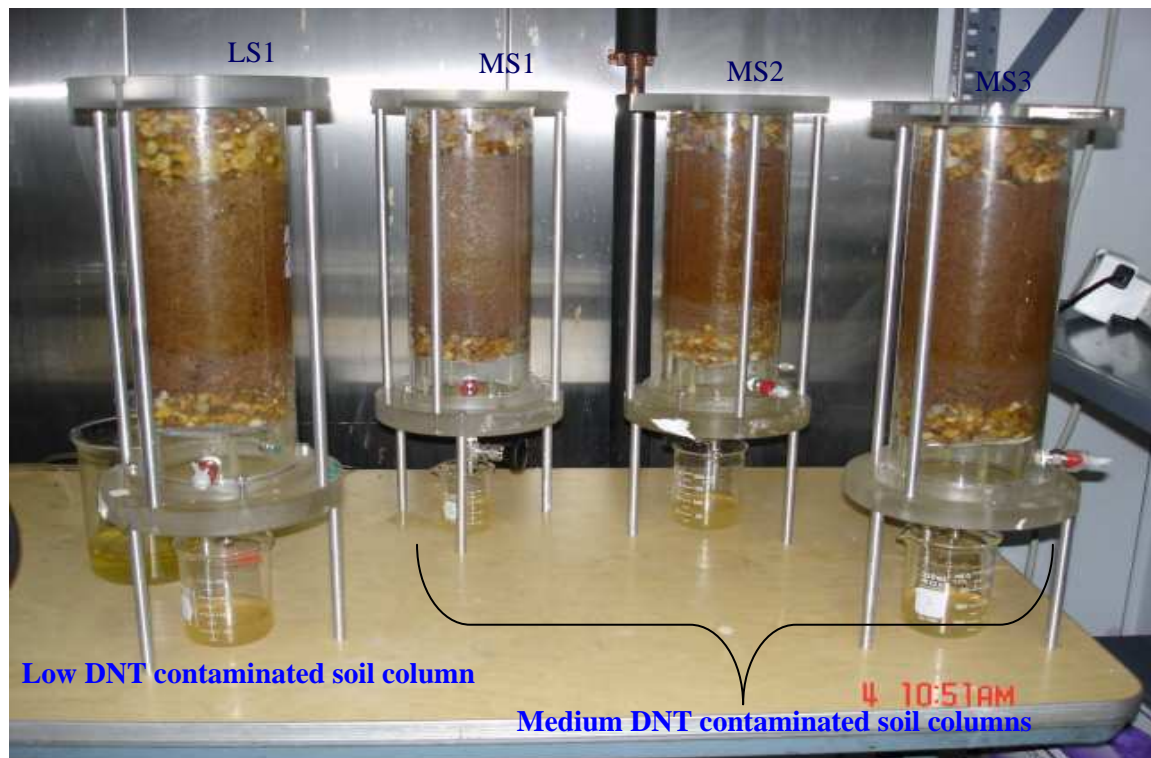
Soil (**MS**) Columns (3EA), and Low Contaminated Soil (**LS**) Column (1EA). The HS was homogenized with a stainless steel spatula and mixed with coarse sand to increase permeability and reduce preferential flow paths. A layer of contaminated soil (1,600 g) was placed over an uncontaminated sand layer (400 g) and the column was left open to the atmosphere. Initially feed solution of 100 mL DI water (without



**Figure 3.1.** Barksdale soil column schematics to assess DNT leaching and degradation in historically contaminated soils.

additional buffer) was added to the top of the column, and the column was not actively aerated (*i.e.*, oxygen supplied through diffusion processes and in feed solution). After 61 days of operation, buffered media was added to the column.

In the other columns (MS and LS columns) (Figure 3.2), phosphate (10 mM) buffered feed solution (100 mL) was applied initially, with a hydraulic retention time (HRT) approximately 3 days based on the breakthrough. MS column studies evaluated the effects of buffer strength, nutrient addition, oxygen availability, and pH on the rate of DNT degradation.



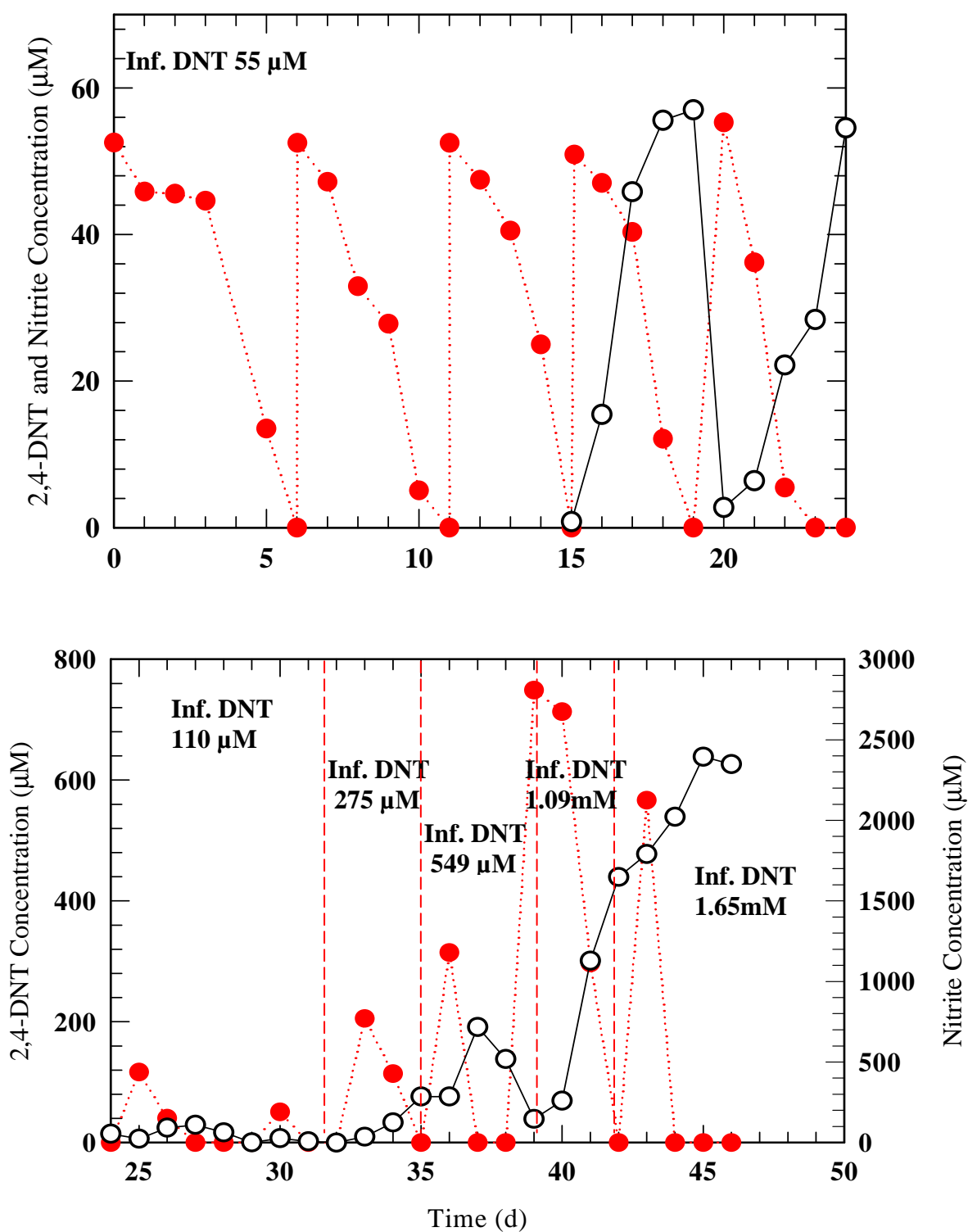
**Figure 3.2.** Soil columns with various DNT background concentrations (LS, Low Contaminated Soil; and MS1, MS2, MS3, Medium Contaminated Soil).

### 3.4. Results and Discussion

#### 3.4.1. *Microcosm Studies for Assessment of DNT Degradation Activity in Barksdale Soils.*

Shake flasks were constructed with soils from the Barksdale sites to assess the potential for 2,4-DNT degradation. Sample names (Lydol Ditch, Trivolen, and Dynamite Area) refer to three site locations within the Barksdale complex. Highly contaminated soils from these three locations were incubated with mineral media. After 7 d of incubation, a small volume was transferred to an Erlenmeyer flask which contained 55  $\mu\text{M}$  2,4-DNT alone. Degradation of 2,4-DNT was observed in all serial transfer flasks from each site. For example, disappearance of 2,4-DNT in the Lydol Ditch sample was observed at 3 days after the initial transfer from the contaminated soil shake flask (Figure 3.3). No lag phase was observed after the initial transfer and degradation proceeded rapidly in transfer flasks where complete degradation of 55  $\mu\text{M}$  2,4-DNT occurred within 4 or 6 days.

Nitrite as indicator was produced in the transfer flasks in concurrence with 2,4-DNT degradation. After 25 days, these cultures were exposed to increasing (stepwise manner) 2,4-DNT concentrations (*i.e.*, 110, 275, 549, 1090 and 1650  $\mu\text{M}$ ), displaying a robust ability to degrade 2,4-DNT. 2,4-DNT degradation in Trivolen and Dynamite samples was observed after 7 and 15 day lag phases, respectively (data not shown). Complete degradation was observed and repeated in serial transfer flasks for a range of contaminant concentrations paralleling Lydol Ditch observation. Nitrite production was observed with stoichiometry of 1.6:1 for  $\text{NO}_2^-$ :DNT.



**Figure 3.3.** Representative shake flask study: 2,4-DNT (●) and  $\text{NO}_2^-$  (○) profiles over time seeded from Lydol Ditch soil.

### **3.4.2. Column Studies: Barksdale Soil Demonstration.**

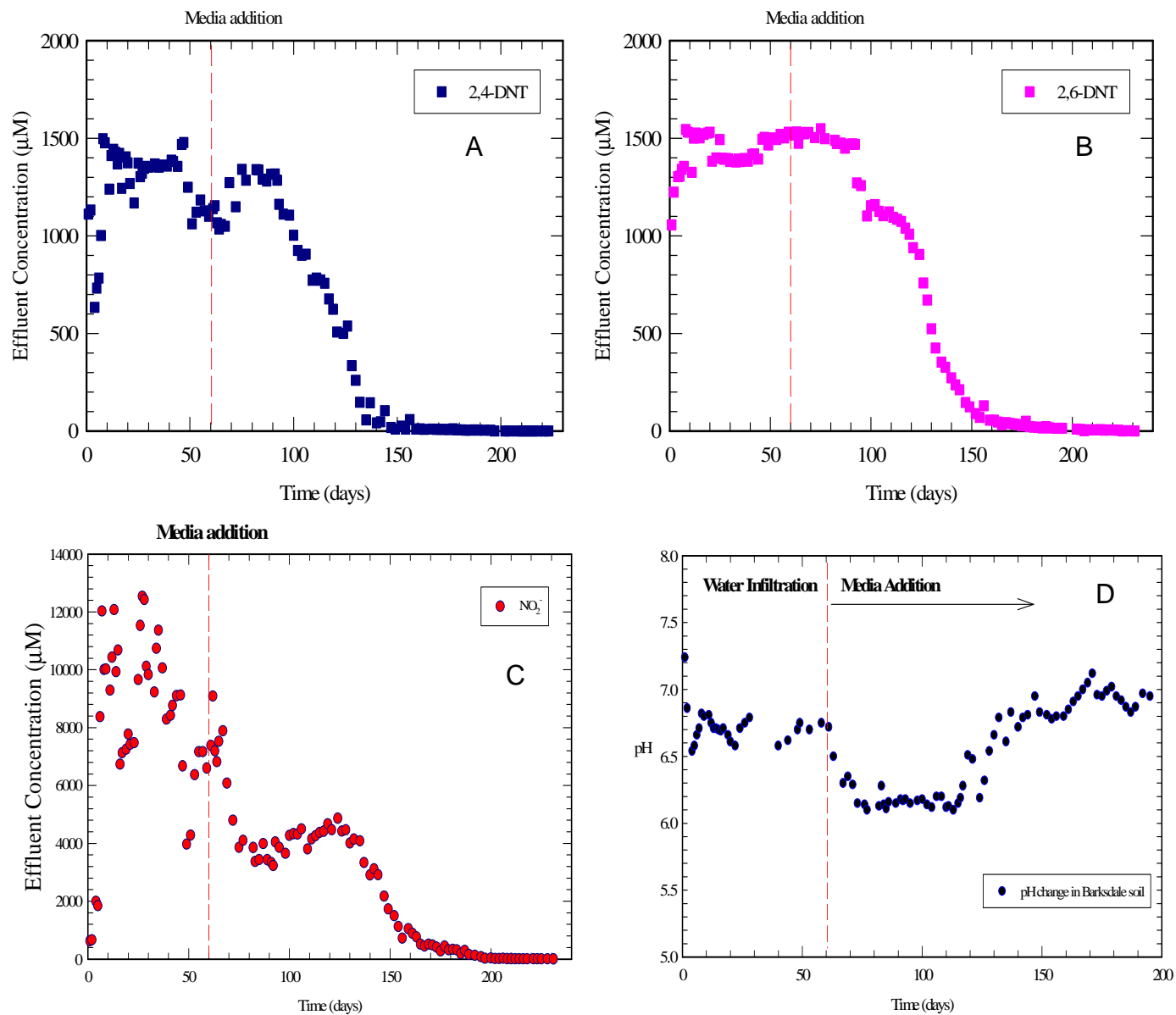
Three sets of columns containing different level of 2,4-DNT (HS, MS, and LS columns) were assembled to better understand the ability of microorganisms in these specific surface soils to degrade DNT and to evaluate the factors involved. Specifically, these studies investigated how low DNT's can be removed through water infiltration and biodegradation, and if 2,6-DNT can be degraded within different DNT's contaminated soils.

#### **3.4.2.1. Highly Contaminated Soil (HS) Column Studies.**

With a feed of 100 mL DI water per day, effluent breakthrough was observed after 2.5 days. HS column effluent samples were analyzed for nitrite, pH, and DNT isomers and the results presented in Figure 3.4. The effluent profiles for DNT were comparable for both isomers (Figure 3.4 A and B). Initially, significant 2,4-DNT concentration was observed in column effluent, and on 8 d after column breakthrough effluent concentrations fluctuated around 1,350  $\mu\text{M}$  for 40 days of operation, gradually decreasing for the following 20 days. Subsequent to media addition, 2,4-DNT effluent concentration increased and reached 1,300  $\mu\text{M}$  for 20 days. Effluent 2,4-DNT level began to decrease and depleted near detection limits by day 150. Similarly, 2,6-DNT concentrations in column effluent were near saturation for 90 days (fluctuating around 1,500  $\mu\text{M}$ ), where no change occurred with media addition. 2,6-DNT effluents near detection limits were eventually observed by day 200.

Nitrite evolution as an indicator of DNT biodegradation was monitored (Figure 3.4C). The initial concentration of nitrite observed in the effluent was approximately 0.6





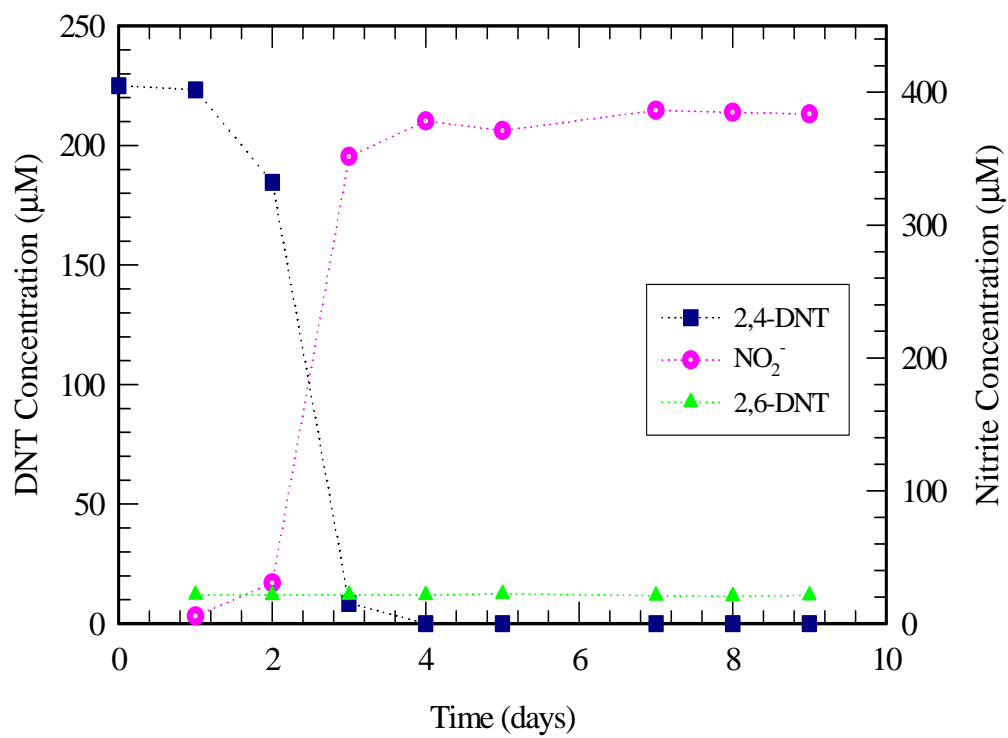
**Figure 3.4.** HS column effluent Profiles: (A) 2,4-DNT effluent concentrations (B) 2,6-DNT effluent concentrations (C)  $\text{NO}_2^-$  effluent concentrations (D) Effluent pH values.

mM and concentrations increased to approximately 1.9 mM after column breakthrough. Over the first 7–10 days a rapid increase of nitrite concentrations were observed, fluctuating around 10 mM. After the addition of mineral media, nitrite effluent concentrations decreased slowly and stabilized around 4.0 mM for 60 days. Nitrite levels decreased again on day 135 and finally approached detection limits on day 200.

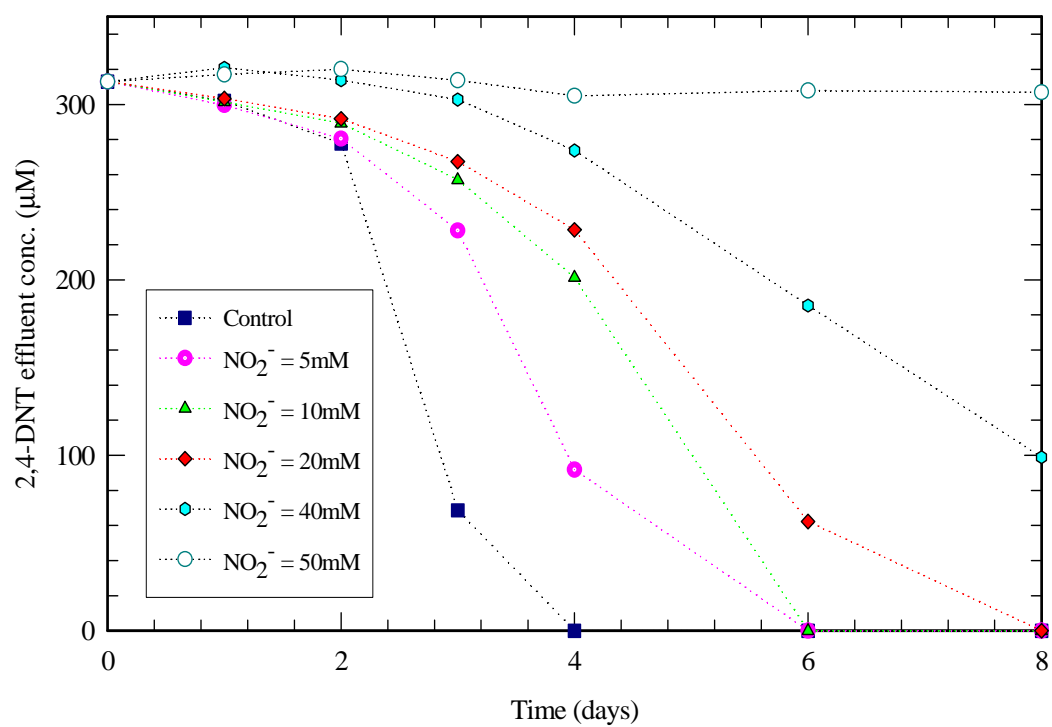
It was necessary to confirm that nitrite production was an indicator of the DNT degradation activity in the column systems. Therefore, shake flask studies containing serial aliquots transferred from HS column effluent were initiated. Results of these studies demonstrated the complete mineralization of 2,4-DNT by the indigenous microorganisms present in the HS (Figure 3.5). Effluent studies taken from day 130 of the column study show rapid depletion of 2,4-DNT (50 mg/L) within 4 days of operation along with a concurrent evolution of nitrite along with a decrease in pH. Degradation of 2,6-DNT, however, was not observed in these microcosm studies.

Further shake flask experiments (containing HS column effluent) were conducted to examine the effects of nitrite accumulation on the activity of the DNT degrading cultures in this specific soil sample. Results indicate that increasing nitrite concentration (from 5 to 50 mM) exerted negative effects on the rate of DNT degradation, with a severe inhibition observed at 50 mM (Figure 3.6). In HS column studies, during the first 60 day nitrite levels fluctuated around 10 mM, which may here slowed DNT degradation.

Nitrate concentrations for several samples were measured and were listed in Table 3.1. Nitrate effluent varied corresponding with varying nitrite effluent, possibly resulting from the presence of nitrifying microorganisms (nitrite convert to nitrate) in the field soils. Nitrate concentrations increased as nitrite concentration reduced. Based on nitrite and



**Figure 3.5.** Shake flask studies. Loss of 2,4-DNT concentrations and consequent nitrite productions in shake flask seeded with HS effluent.



**Figure 3.6.** Loss of 2,4-DNT under various initial concentrations of nitrite (seed source: HS effluent shake flask).

**Table 3.1.** Nitrite, Nitrate, SN, and N-ratio ( $\text{NO}_3^-/\text{NO}_2^-$ ) in the HS column effluents.

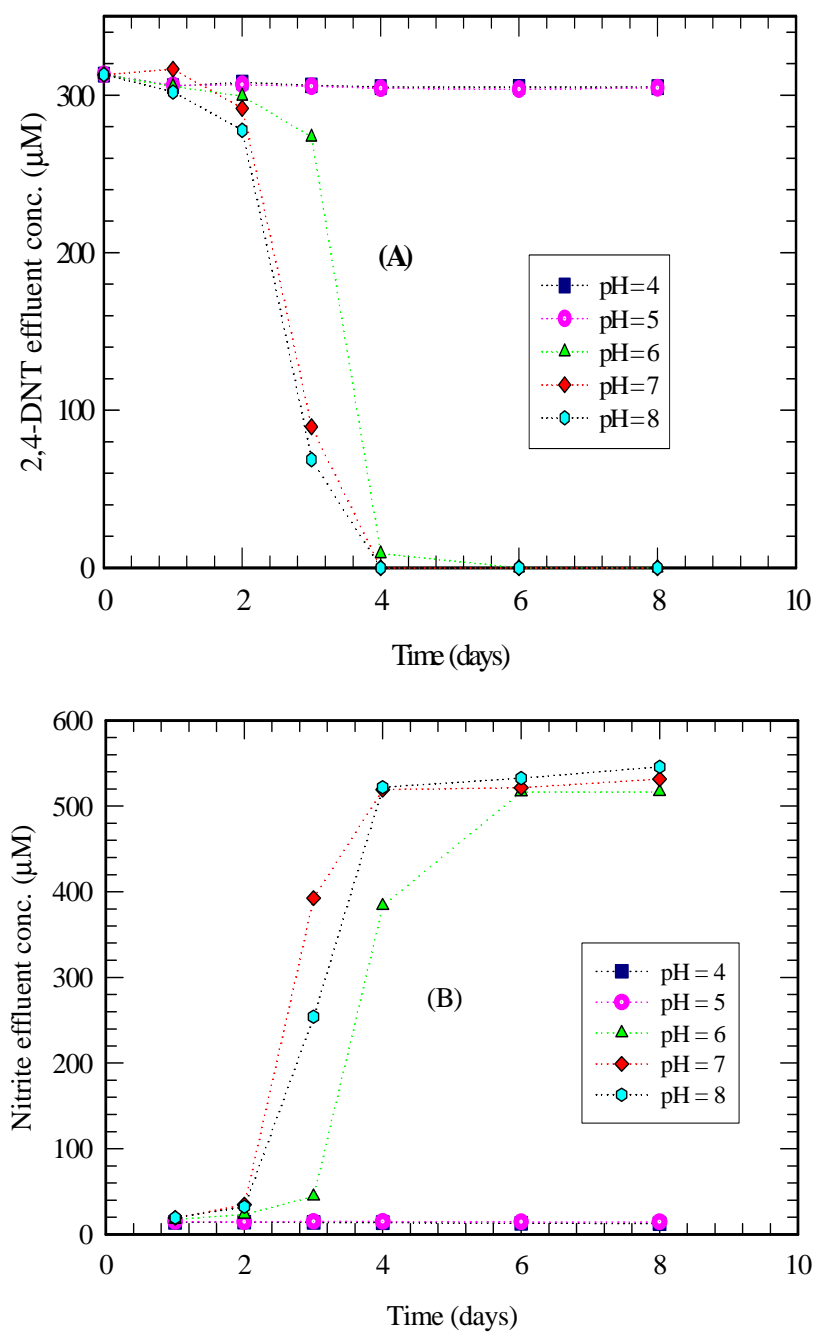
	days	Eff. Nitrite ( $\mu\text{M}$ )	Eff. Nitrate ( $\mu\text{M}$ )	SN	N-ratio
	57	7,962	352	8,315	0.04
<b>Media addition</b>	61	7,986	358	8,344	0.05
	63	7,404	357	7,761	0.05
	91	4,580	372	4,952	0.08
	132	3,792	306	4,098	0.08
	135	3,587	293	3,880	0.08
	147	1,813	891	2,704	0.49
	149	1,403	937	2,340	0.67
	165	540	827	1,367	1.53
	167	421	747	1,,168	1.77
	192	152	748	900	4.94
	195	115	912	1,027	7.95

(Eff.): Experimental effluent concentration

nitrate production, sum of nitrogen (SN) and  $\text{NO}_3^-/\text{NO}_2^-$  (N-ratio) were calculated, which resulted over time in decrease of SN and increase of N-ratio (Table 3.1). Despite considerable loss of DNT due to washout and biodegradation, considerable SN (appreciable nitrite and relatively high amounts of nitrate) were evolved, indicating that DNT was still degrading in the column despite the low effluent concentration.

The effluent pH values ranged from 6.5 to 7.0 during water infiltration events, indicating that the soil contained sufficient buffer capacity for DNT degradation (Figure 3.4D). The effluent pH decreased subsequent to buffered media addition (60 d) and stabilized around pH 6.1. As washout and degradation lowered the total DNT remaining in soil, pH eventually increased (day 120) to approximately pH 7 (Figure 3.4D). Increasing DNT degradation causes pH drop. However, it is impossible to speculate the effect of media addition on the rate of biodegradation based on this observation, as SN (nitrite + nitrate) production decreased inconsistently during this period. The pH drop can possibly result from washout of buffer capacity in column, not an increase in DNT degradation.

The pH drop can also cause inhibition of DNT biodegradation. To examine inhibitory pH values on the activity of the DNT degrading cultures, additional shake flask study was conducted. Results indicate that pH values of 6.0 and below can be inhibitory to the 2,4-DNT degrading cultures, thus restricting the mineralization in contaminated soils (Figure 3.7). During HS column studies, effluent pH values were relatively stable and appropriate for DNT degraders (between 6.0 and 7.0). This indicates that DNT was biodegraded in the soil column system without concerns of pH inhibition.



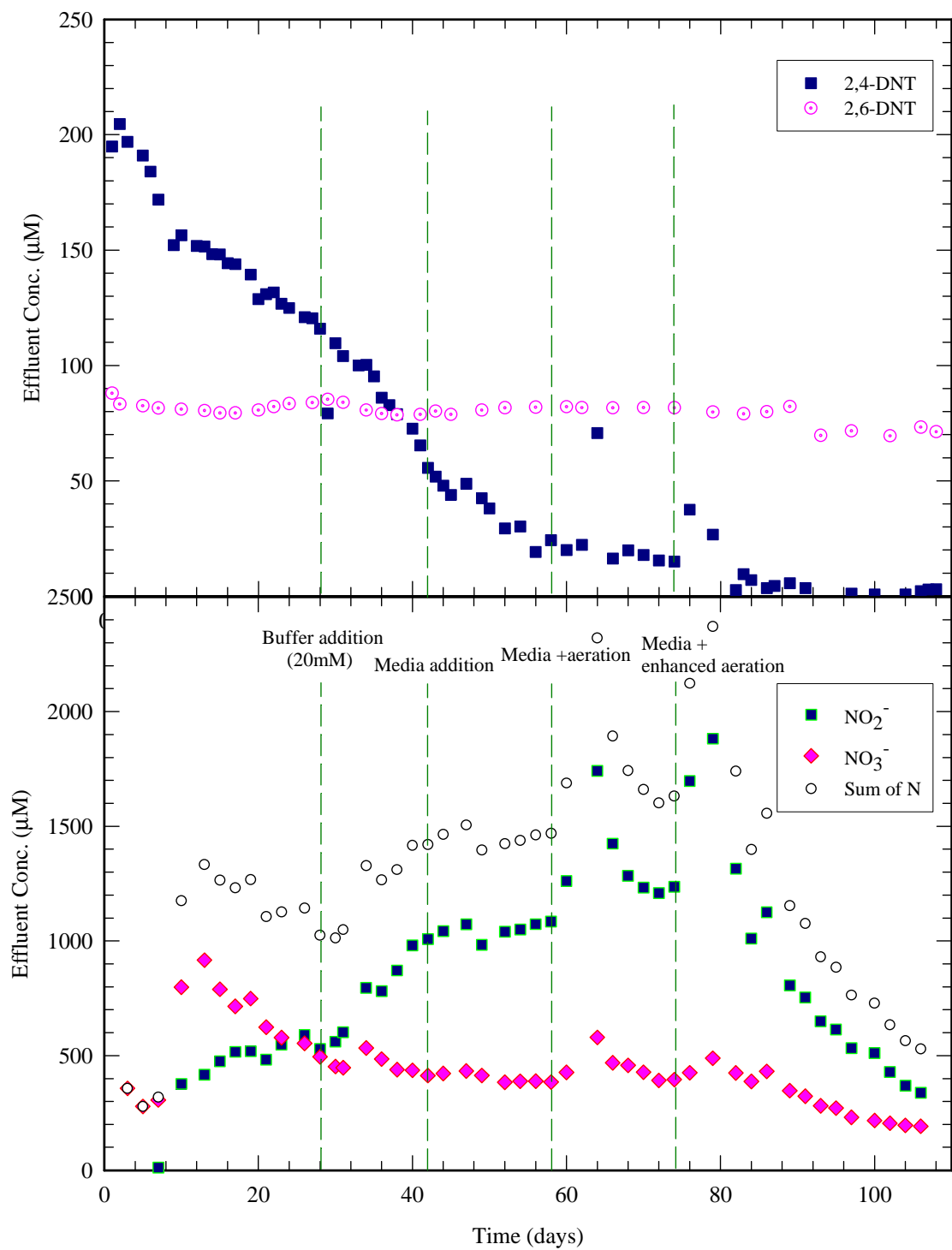
**Figure 3.7.** Changes in 2,4-DNT concentrations (A) and nitrite production (B) under varied pH conditions (seed source: HS effluent shake flask).

#### **3.4.2.2. Medium Contaminated Soil (MS) Column Studies.**

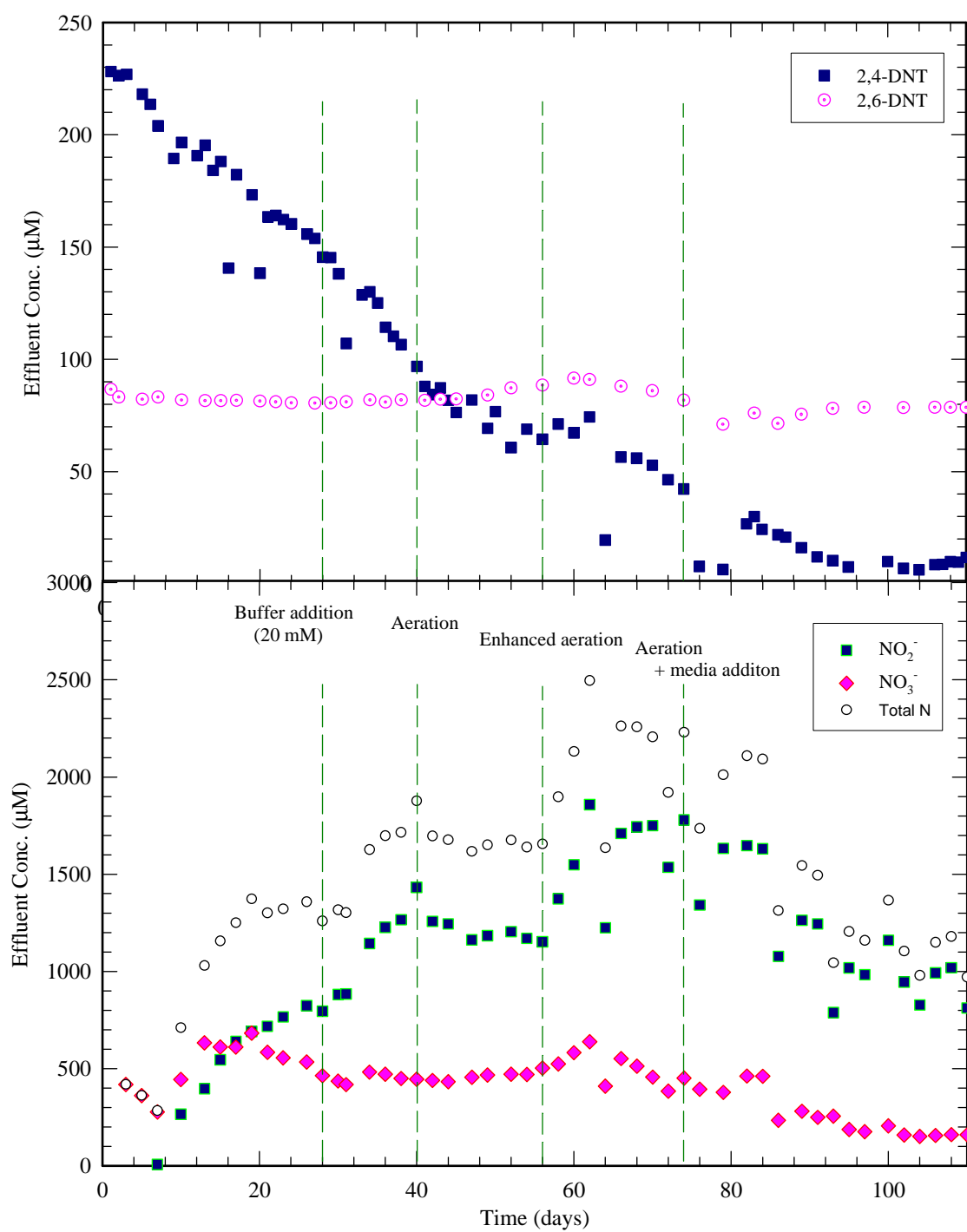
Subsequent column studies involved soil columns containing near saturation 2,4-DNT concentrations ( $1,145 \pm 151$  to  $1,431 \pm 101$  mg/kg soil). Three identical columns were employed to assess how buffer strength, media addition, enhanced aeration, and pH influence biodegradation capacity in surface soils. For all MS columns, initial effluent pH was low (between pH 4 and 6), which can lead to the cessation of degradation activity even though the processes started with 10 mM phosphate buffer strength. Nevertheless, measurable SN was produced over the first 28 days of operation. Amendment with 20 mM buffer concentration led to suitable effluent pH and corresponding stable and higher concentration of nitrite.

In Figure 3.8 (MS1 column), results present that there was no increase of SN after media addition, while humidified air (saturated) delivered to the column caused increase of SN and thus enhanced aeration was provided. However, it could not evaluate the effect of additional aeration because leaching 2,4-DNT and SN were low due to biodegradation and washout. Following 80 d of operation, 2,4-DNT effluent concentration decreased and eventually dropped near detection limits. MS2 column was established to better understand the effect of oxygen availability (Figure 3.9). Based on SN profiles, initial periodic aeration did not affect the rate of biodegradation, but more frequent aeration caused slightly increase of SN concentration (*i.e.* DNT degradation). These results indicate that no observable DNT degradation was attributed to nutrient limitation in this particular contaminated soil, but oxygen availability slightly influenced DNT degradation.





**Figure 3.8.** MS1 column effluent profiles: 2,4-DNT and 2,6-DNT, and nitrogen ( $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , and Sum of Nitrogen) concentrations.



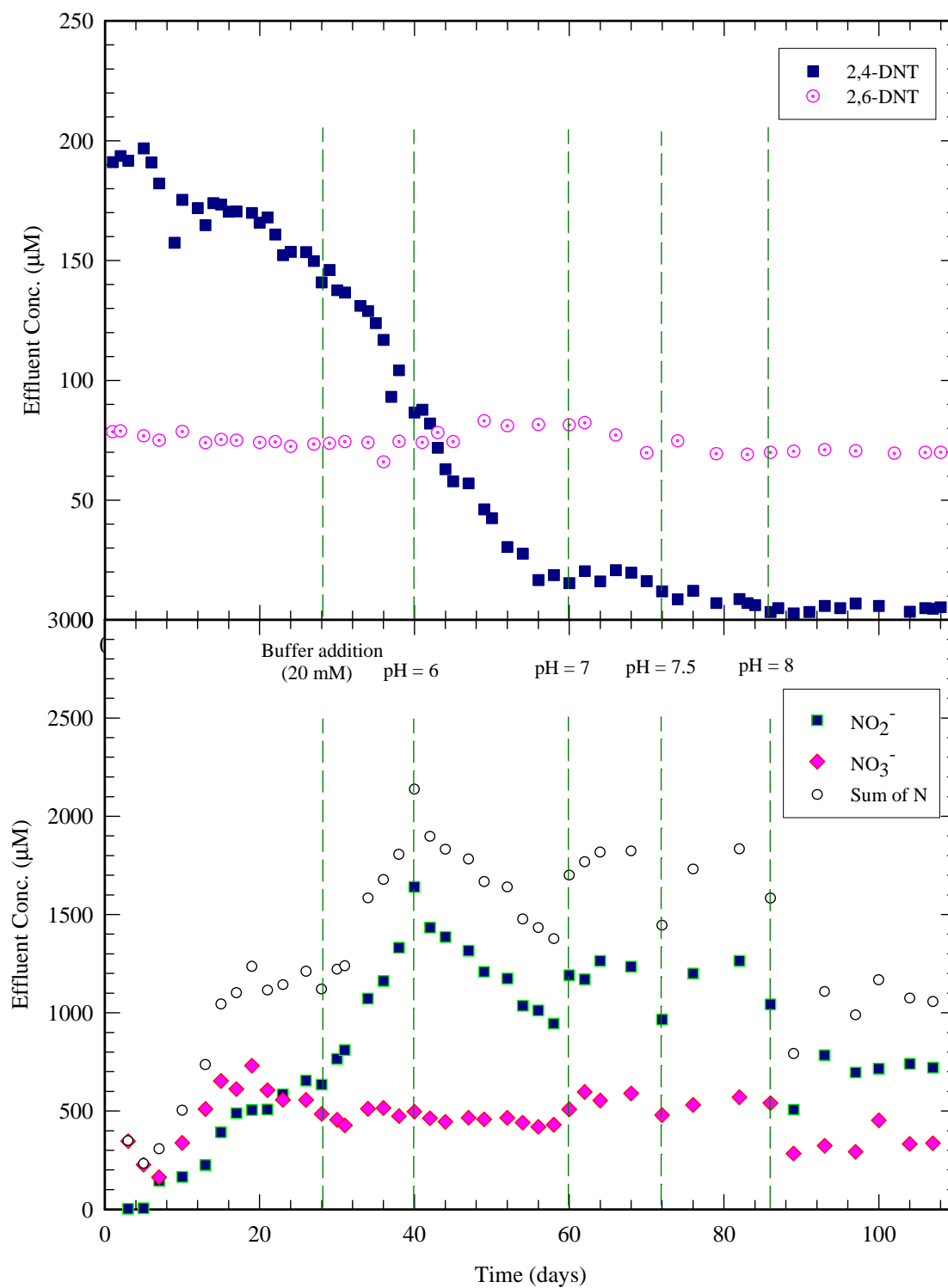
**Figure 3.9.** MS2 column effluent profiles: 2,4-DNT and 2,6-DNT, and nitrogen ( $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , and SN) concentrations.

MS3 column was operated to evaluate the effect of influent pH. The results (Figure 3.10) suggest that the rate of DNT degradation was limited at pH value of 6.0. At a pH 6 in feed solutions, a decrease of SN was observed (compared to an initial pH value of 8.3). As the influent pH increased to 7.0 and 7.5, SN levels increased slightly and stabilized. Consistently, immediate reduction of SN level was observed on day 85 due to system-wide 2,4-DNT depletion.

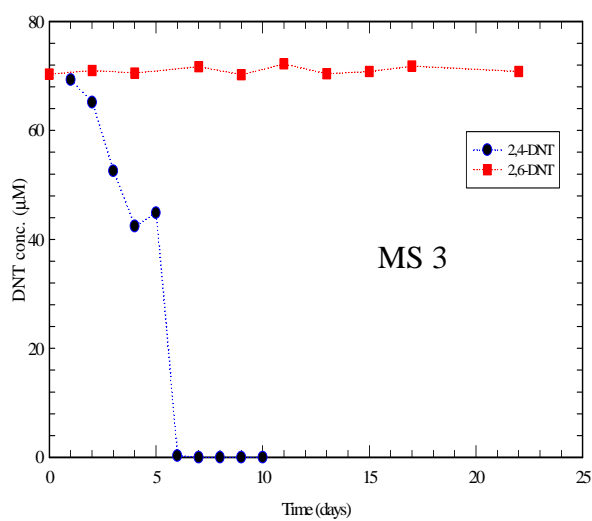
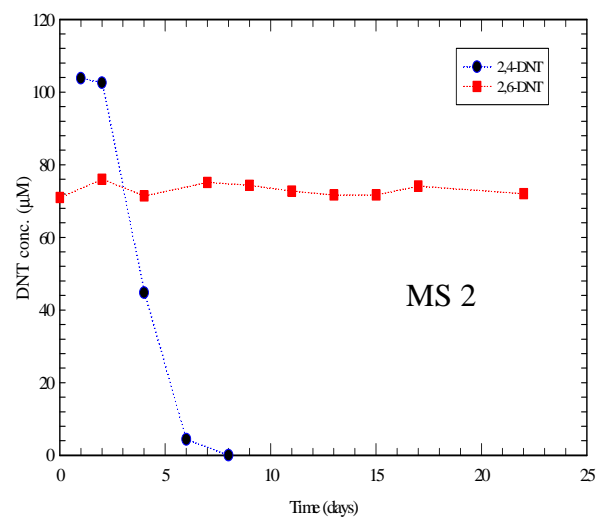
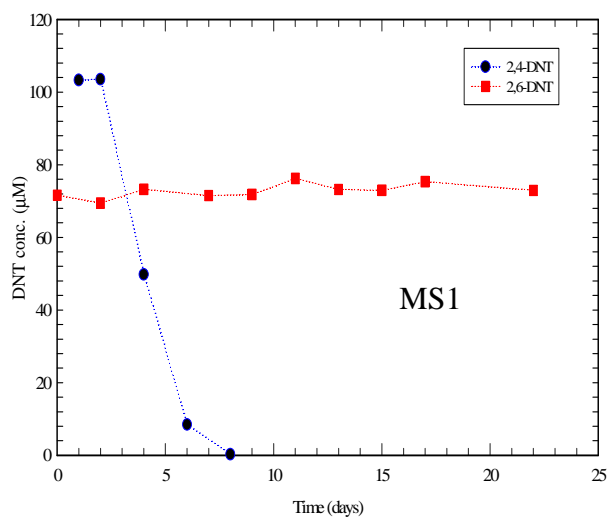
Shake flask experiments were conducted with column effluents as accurate indicator for DNT degradation activity. Figure 3.11 presents the loss of 2,4-DNT in MS column effluent shake flasks. The complete loss of 2,4-DNT was observed after 6 or 8 day of incubation. No depletion of 2,6-DNT was observed at any shake flasks.

#### **3.4.2.3. Low Contaminated Soil (LS) Column Studies.**

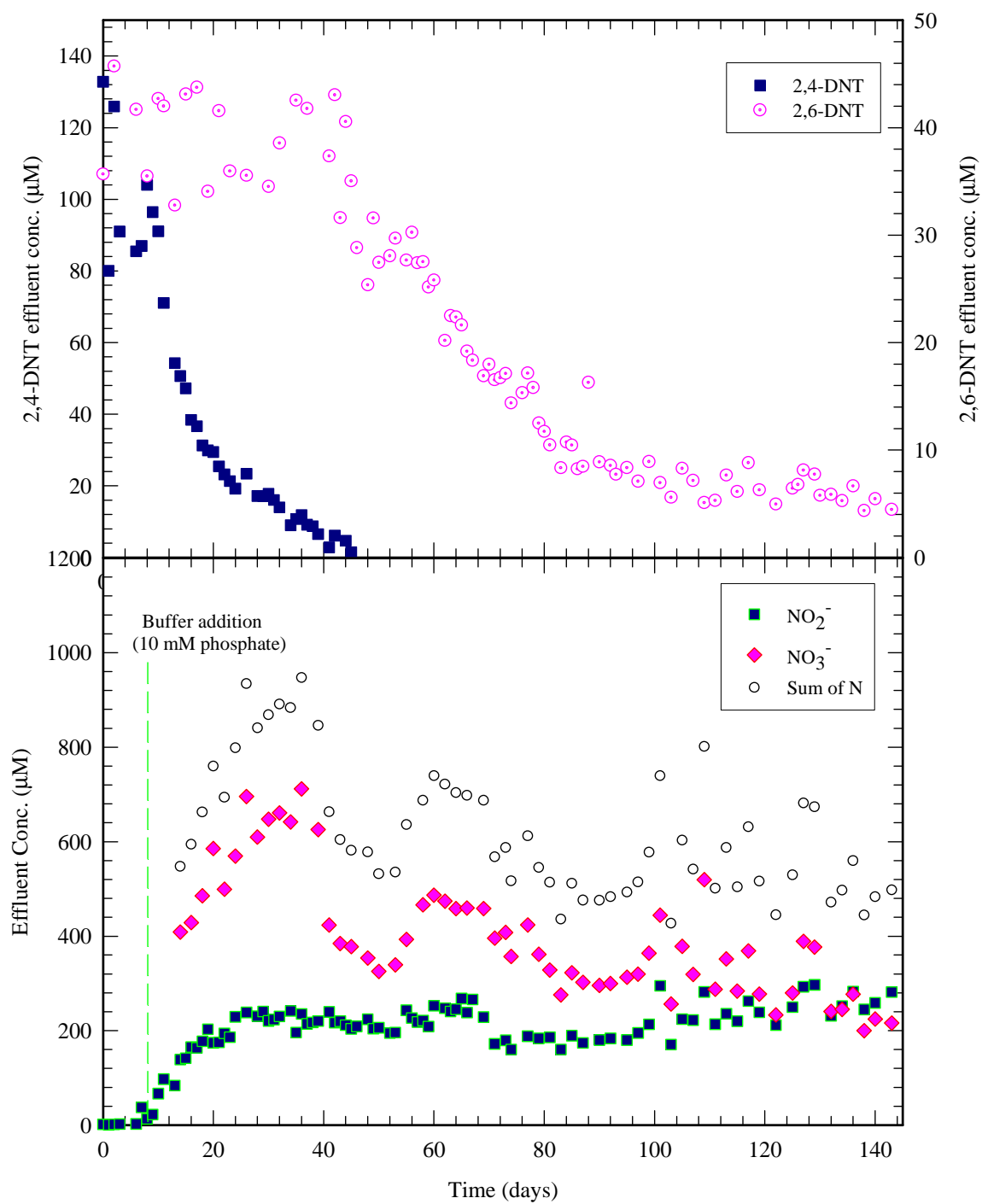
An additional bench scale column was operated to investigate DNT degradation within relatively low contaminated soil ( $728 \pm 509$  mg/kg soil for 2,4-DNT). Figure 3.12 presents leaching concentration of DNT isomer and associated nitrogen concentration (*i.e.*, nitrite, nitrate, and SN). During the first 11 days of operation, the pH value of effluent ranged from 6.2 to 6.7. After 10 mM phosphate buffer was added, the values of pH increased to 6.9 and above. Under suitable pH conditions, 2,4-DNT effluent concentration decreased and corresponding SN concentration increased to approximately 1,000  $\mu$ M. On day 45, 2,4-DNT effluent concentration decreased below detection limits. Effluent 2,6-DNT levels fluctuated (35 to 45  $\mu$ M) during the first 40 days of operation. Interestingly, 2,6-DNT effluent concentrations began to decrease consequent with depletion of 2,4-DNT. Despite complete depletion of 2,4-DNT considerable SN



**Figure 3.10.** MS3 column effluent profiles: 2,4-DNT and 2,6-DNT, and nitrogen ( $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , and Sum of N) concentrations.



**Figure 3.11.** DNT profiles in MS1, MS2 and MS3 effluent shake flasks (taken at day 30, 30, and 40 respectively).



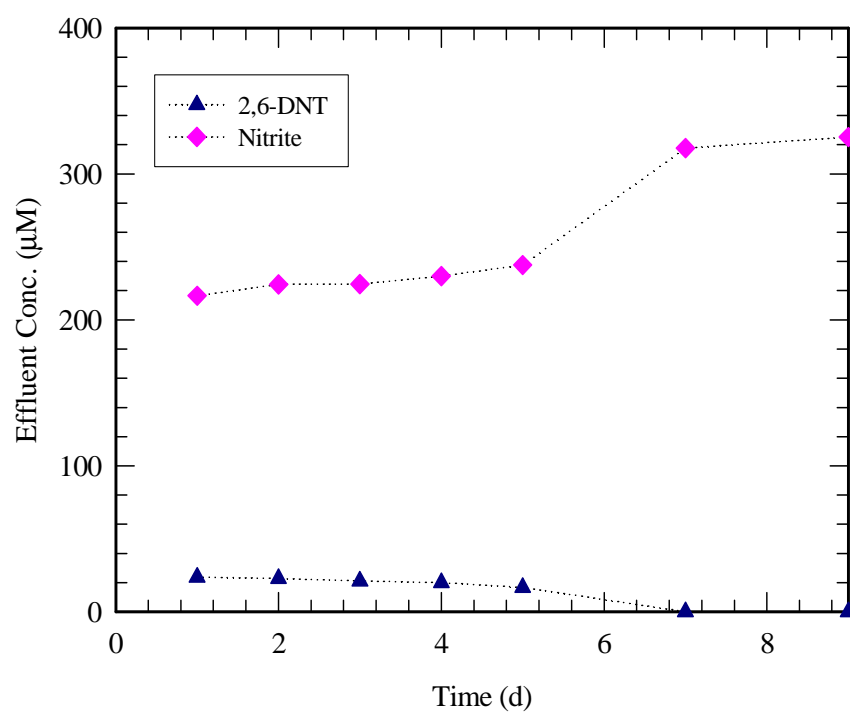
**Figure 3.12.** LS column effluent profiles: 2,4-DNT, 2,6-DNT, and nitrogen ( $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , and Sum of N) concentrations.

fluctuated above 400  $\mu\text{M}$ , indicating SN production attributed to 2,6-DNT biodegradation. After 45 d of operation, 2,6-DNT effluent concentration decreased to 5  $\mu\text{M}$  and considerable SN was still produced.

To confirm 2,6-DNT degradation occurring after depletion of 2,4-DNT, shake flask studies were established with column effluent by day 100. Figure 3.13 indicates that occurrence of 2,6-DNT degradation was observed from complete loss of 2,6-DNT (42  $\mu\text{M}$ ) and observable increase of nitrite over 9 days. This indicates that 2,6-DNT degradation capacity was eventually present (enriched).

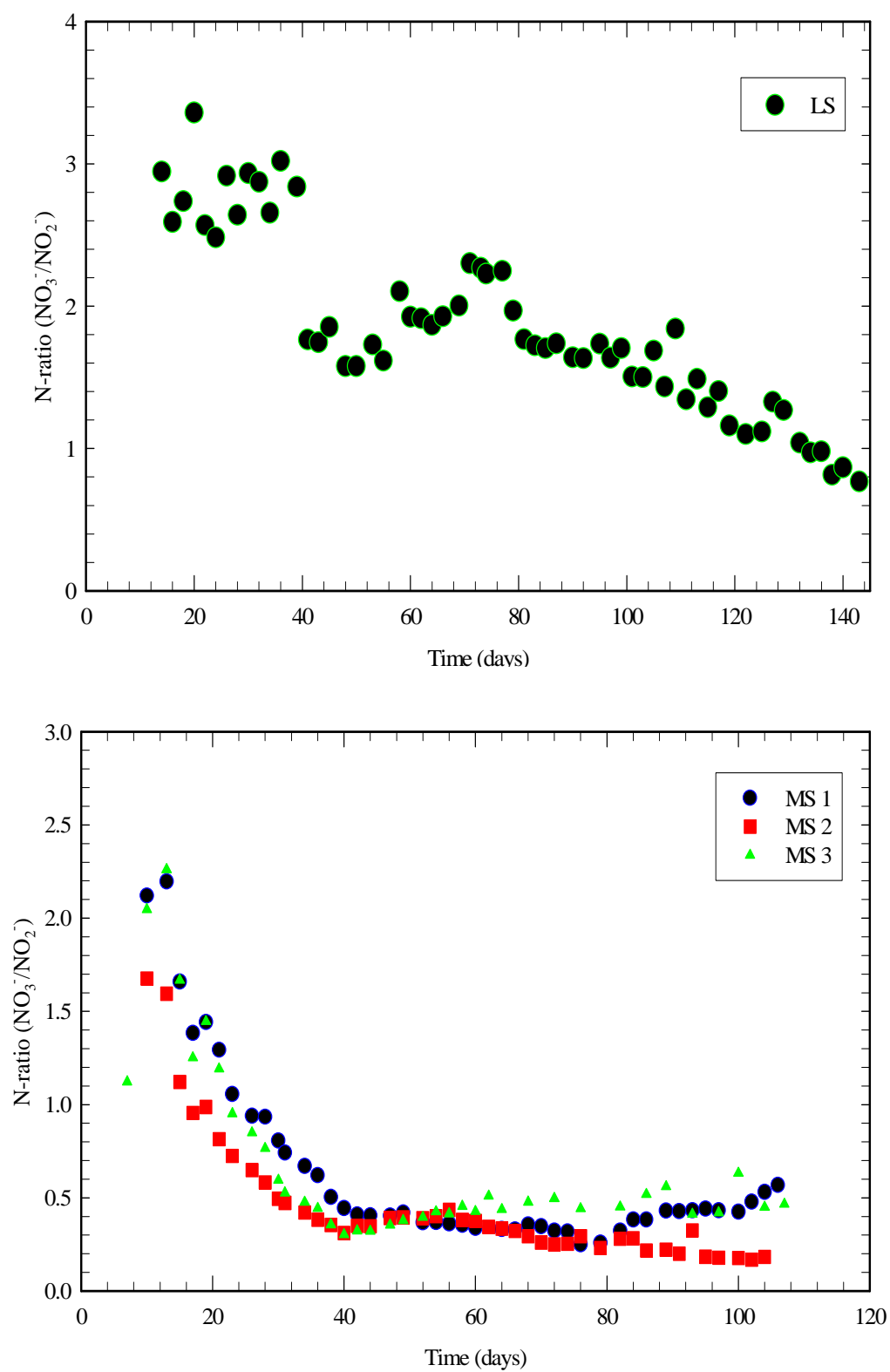
The presence of both  $\text{NO}_2^-$  and  $\text{NO}_3^-$  were important consideration for assessment of biodegradation of DNT. N-ratio ( $\text{NO}_3^- / \text{NO}_2^-$ ) for LS column was observed above 1.0 because nitrate effluent concentrations were higher than nitrite concentrations (Figure 3.14A). In contrast, MS column systems (each being similar) displayed N-ratio decreasing in stepwise manner, eventually stabilized below 1.0 on 40 d of operation (Figure 3.14B). As a result, the N-ratio was controlled by DNT levels in the soils, indicating useful tool to predict biodegradation at varying DNT contaminated sites.

At the completion of the column studies, soil samples were analyzed to identify remaining DNT concentrations and compared with background concentrations prior to operation. In the HS system, considerable DNT isomers were removed, but soils contained considerable amounts (Data not shown). MS column experiments displayed a high mass removal of 2,4-DNT (approximately 94–96 %), but 2,6-DNT was minimally removed (approximately 16–19 %) (Table 3.2). The LS column demonstrated the relatively higher removal of both 2,4-DNT and 2,6 DNT with  $\geq 99$  % and approximately 66 % respectively.



**Figure 3.13.** 2,6-DNT degradation and nitrite production in LS column shake flasks containing effluent taken by 100 d.





**Figure 3.14.** The ratio of nitrate to nitrite produced in (A) LS and (B) MS column systems.

**Table 3.2.** Soil extraction results; DNT concentrations remained after treatment vs. background DNT concentrations.

Column	Background concentration (mg/kg)		Remaining concentration After 150 days (mg/kg)		% removal (2,4-/2,6-DNT)
	2,4-DNT	2,6-DNT	2,4-DNT	2,6-DNT	
MS 1	1,431±101	101±101	57±35	82±21	96 / 19
MS 2	1,431±101	101±101	91±41	85±17	94 / 16
MS 3	1,145±151	82±87	58±45	59±23	95 / 19
LS	728±509	42±31	trace	14±3	≥ 99 / 66

### 3.5. Conclusions

In Fortner's column studies (1) conducted with Badger Army Ammunition Plant (BAAP) soils, complete nutrient limitations controlled the onset of 2,4-DNT degradation. Despite rapid 2,4-DNT degradation, the less amounts of 2,4-DNT in soils were removed through washout and biodegradation. Biodegradation of 2,6-DNT was not, however, observed under any conditions. While high amounts of nitrite were produced, appreciable nitrate production was not observed during the study.

For Barksdale soils (herein), phosphate-buffered deionized water alone was sufficient to induce or enhance 2,4-DNT bioactivity. This suggests that no observable increase of 2,4-DNT degradation was attributed to nutrient addition, but phosphate may be critical as a nutrient to sustain degradation for long time periods. The 2,4-DNT degradation rapidly occurred in the infiltration column systems, and the high percent of 2,4-DNT was removed in the soils. This suggests that low endpoint of 2,4-DNT via biodegradation can be expected in surface soil without concerns of nitrite toxicity and pH inhibition before leaching to vadose zone. However, 2,6-DNT mineralization was limited by extent of 2,4-DNT contamination in surface soils. The recalcitrant nature of 2,6-DNT can be expected at highly contaminated soils, while 2,6-DNT biodegradation can be initiated after depletion of 2,4-DNT at relatively low DNT contaminated soil. This column study also demonstrated that nitrifying bacteria can be active under appropriate conditions (*i.e.*, sufficient buffer and nutrient), producing considerable nitrate. If efficient nitrification in surface soil can be accomplished, the probability of *in situ* bioremediation being a remediation strategy for DNT contamination sites may increase as nitrite toxicity will be minimized.

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## **CHAPTER 4**

### **Monitoring Performance and Factors Controlling DNT Degradation in Vadose-Zone Simulations**

#### **4.1. Overview**

This chapter presents findings regarding the effects of environmental factors on the biodegradation of DNT in vadose zone during infiltration due to bioremediation or natural leaching. Experiments were conducted in bench scale column studies, exploring variables including pH and nitrite concentration with the goals of quantifying and enhancing DNT mineralization activity.

The rapid biodegradation of both DNT isomers was observed in initial column studies which were bioaugmented with previously enriched cultures at DNT concentrations near saturation ( $\sim 1000 \mu\text{M}$ ). Hydraulic retention time (HRT) influenced DNT biodegradation, but enhanced aeration did not. Evolved nitrite was detected, but at less than stoichiometric amounts due to biological nitrification processes converting nitrite to nitrate. Additional column studies indicated that solution pH becomes inhibitory at pH of 5.5 for both 2,4-DNT and 2,6-DNT degrading mixed cultures. While the pH effect could be reversed for 2,4-DNT degrading systems by raising the influent pH, the 2,6-DNT recovery was slow and unpredictable, suggesting that pH inhibition may have extended effects on 2,6-DNT degradation processes. Additionally, the presence of nitrite oxidizers in the soil decreased the potential negative effect of nitrite on DNT degrading mixed cultures.

## 4.2. Introduction

Bioremediation of contaminated soil through infiltration with water and amendments will result in the transport of DNTs into the underlying vadose zone. An important concern that emerges is whether natural attenuation (NA) in the underlying porous media will occur at rates that prevent the contaminants from reaching the saturated zone. There are many factors that may influence DNT NA in vadose zone including availability of oxygen, availability of nutrients, and bi-products of biodegradation (1).

The work that forms the foundation of *in situ* biodegradation of DNT contaminated soil is the study conducted by Fortner *et al.* (1), which employed soil column and respirometer studies using contaminated soils from Badger Army Ammunition Plant (BAAP). However, no direct attempt was made to evaluate the rate of leached DNT into “clean” underlying soils or the degradation extent that can occur there in. Important factors to consider in an underlying media include low pH, high nitrite concentrations, oxygen availability, nutrient limitations, *etc.* that can adversely influence degradation of DNT (2,4-DNT and 2,6-DNT) within vadose zone soils. Also, the water infiltration rate may be an important factor influencing the biodegradation of DNT in vadose zones due to bioremediation systems.

Nitrite is evolved during degradation of DNT and its production has been used as an indicator of DNT biodegradation (1-8). However, nitrite can be converted to nitrate by nitrifying bacteria (9-12). Nitrite is usually found only in trace amounts in aerobic habitats and only accumulates at low oxygen partial pressures (9). Under aerobic conditions such as those needed for DNT oxidation, nitrite is converted to nitrate. High

DNT levels can inhibit nitrification directly or indirectly. But when DNT is present at low levels, nitrate formation is likely, and nitrite production may not be as useful indicator for *in situ* biodegradation. In vadose zones, the aerobic conversion of nitrite to nitrate has not been examined under conditions representative of those expected in DNT bioremediation.

This study evaluated the influence of various environmental factors (*e.g.*, retention time (RT) and oxygen availability, pH and nitrite) on the rate and extent of DNT biodegradation in vadose zone soils receiving water infiltration. The threshold values of pH and nitrite concentration which inhibit DNT degradation were estimated through column and batch studies. Additionally the use of nitrite as a conclusive indicator of DNT biodegradation was examined through screening the activity of nitrite oxidizers in the soils.

### **4.3. Materials and Methods**

#### **4.3.1. Chemical and Material Sources.**

Primary isomers of DNT, 2,4-dinitrotoluene (97 %) and 2,6-dinitrotoluene (98 %), were purchased from Sigma-Aldrich. Reagent grade chemicals for mineral medium were purchased from Fisher Scientific and Acros. Other analytical standards and all organic solvents were described in Chapter 3.

The sources for materials used in these column studies were: clean sand from U.S. Silica (Ottawa, Illinois), coarse sand from Staples, 0.145 cm wire mesh cloth from McMaster (Atlanta, GA), 0.64 cm ball valve and inlet valve from Swagelok (Niagara Falls, Ontario).



#### **4.3.2. Mineral Medium Preparation.**

The mineral media used were identical to that described by Spanggard *et al.* (8) and contained the following composition:  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (50 mg/L),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (3 mg/L),  $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$  (100 mg/L), NaCl (500 mg/L),  $\text{H}_3\text{BO}_3$  (100 mg/L),  $\text{CaSO}_4 \cdot 5\text{H}_2\text{O}$  (50 mg/L),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (50 mg/L),  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (50 mg/L) and variable phosphate ( $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ ) buffer strength. NaOH (1N) was used to adjust pH (8–8.5) of the mineral media as needed.

#### **4.3.3. Analytical Methods.**

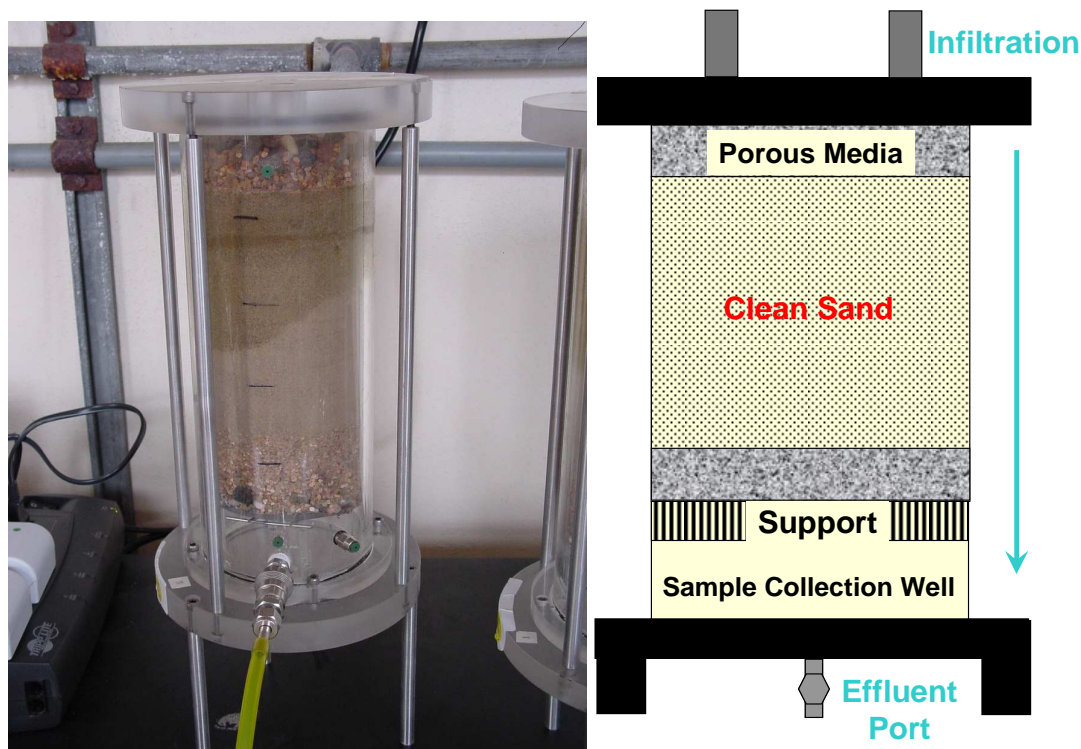
Aqueous samples were analyzed by HPLC (HP Series 1100, Agilent) on a Zorbax® SB-C18 reverse phase column (13) or Hybercarb® porous graphite column (14) using protocols mentioned in Chapter 3 (1). Standard Methods were adopted for the measurement of nitrite and nitrate using spectrophotometer and IC (14).

#### **4.3.4. Vadose Zone Simulated Column Studies.**

Column studies were conducted using bench scale columns designed by Fortner *et al.* (1). Columns were first filled with porous support gravel (3.5 cm) on the wire mesh (0.145 cm), followed by a clean sand (Flint Silica # 12 Ottawa, IL), and a layer of gravel (7.5 cm) (Figure 4.1). A feed solution (50 to 100 mL) consisting of nearly saturated concentrations of DNT (approximately 1000  $\mu\text{M}$ ) of an individual DNT isomer dissolved in media was applied to the columns daily, and effluent was collected from the bottom of the columns to quantify pH, DNT, nitrite, and nitrate. Columns were aerated daily with

humidified and ambient air at 26.6 mL/min for 20 min (total of 532 mL). Columns were inoculated with the 2,4-DNT and 2,6-DNT degrading mixed cultures retaining for 1 day with 200 mL aliquots (enriched from contaminated soil described by Fortner *et al.* (1) and from Tyndall AFRL, respectively) in media buffered to a pH = 8.0.

To investigate the effect of RT, the infiltration rate was varied to create a RT of approximately 3 to 7 days. Additional column experiments assessed the effect of enhanced aeration with increased aeration period (*i.e.*, intermittent aeration, 532 mL per 3 or 2 hr). Subsequent column studies evaluated effects of influent medium composition (*i.e.*, initial pH and nitrite). These experiments evaluated the threshold values of bi-products (pH and nitrite) that inhibit DNT degradation. Table 4.1 presents experimental protocol designed with various parameters.



**Figure 4.1.** Schematics of bench scale simulation of vadose zone to assess degradation of DNT in this area.

**Table 4.1.** Soil column studies to assess the impact of pH and nitrite: Design matrix

Parameters		Range Tested	
		2,4-DNT	2,6-DNT
	<b>Medium</b>	Mineral medium	
<b>Baseline</b>	<b>Buffer</b>	10 mM Phosphate Buffer	
	<b>pH</b>	8.0, maintained with addition of 0.1 N NaOH	
	<b>pH</b>	Initial pH ranging from 8.0 to 5.5 at an interval of 0.5	Initial pH ranging from 9.0 to 5.5 at an interval of 1 or 0.5
	<b>Nitrite</b>	Initial nitrite concentrations ranging from 15 to 70 mM at an increment of 5 or 10 mM	Initial nitrite concentrations ranging from 10 to 130 mM at an increment of 5, 10, or 20 mM

#### **4.3.5. Screening Activity of Nitrifying Bacteria.**

Shake flasks were prepared using the collected wet soil sample (5 g) taken from column and mineral medium (50 mL) with external nitrite spiked. After 7 days incubation, aqueous samples (1mL) re-suspended from initial shake flasks were transferred to soil free microcosms containing fresh medium and 2,4-DNT (1,000  $\mu$ M) to evaluate the presence of specific nitrite oxidizer (*i.e.*, nitrate production). pH in shake flasks was adjusted at 8.0. Once a nitrifying culture was enriched from column, factors (*i.e.*, low pH) inhibiting nitrification were investigated. pH was adjusted at 6.0, and nitrite/nitrate were monitored. All shake flasks were incubated at room temperature on a shaker at 150 rpm.

### **4.4. Results and Discussion**

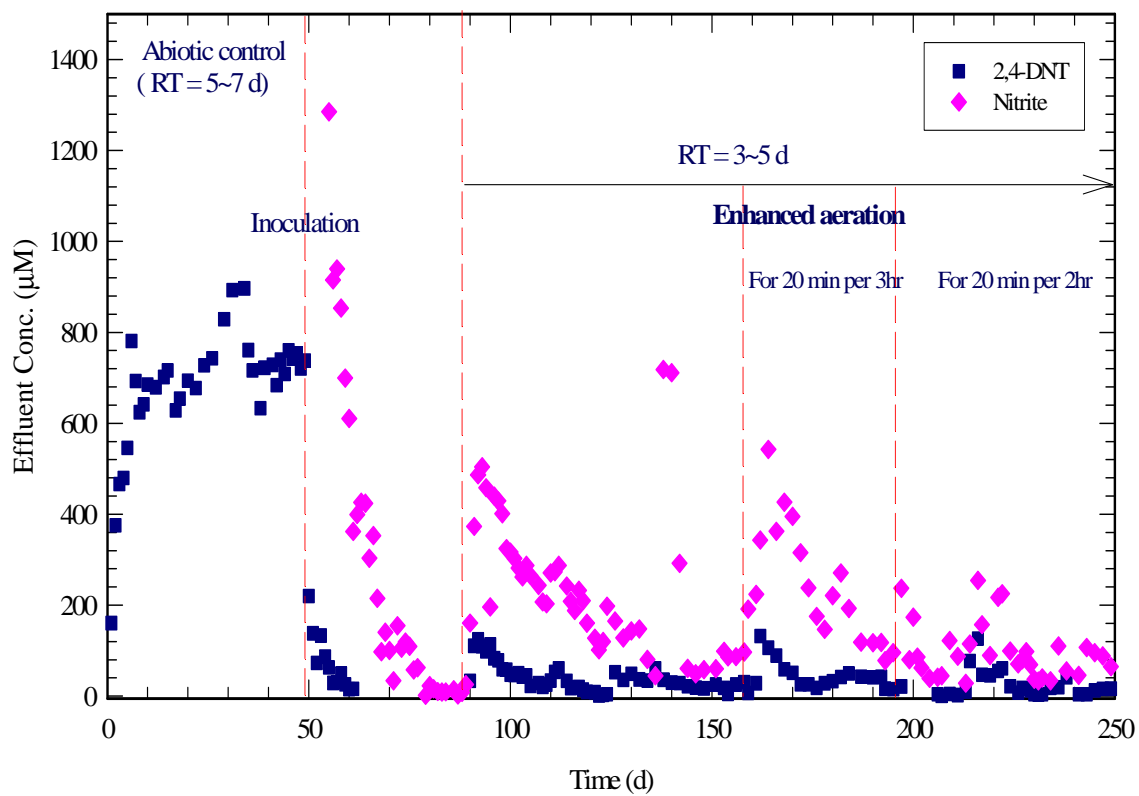
#### **4.4.1. Vadose Zone Simulations: Initial Column Studies.**

Duplicate column systems were conducted to examine DNT degradation under conditions which simulated a vadose zone undergoing infiltration. Column inputs included a single isomer of DNT (approximately 1000  $\mu$ M), nutrient media, and aeration. Column effluent data are presented in Figures 4.2 and 4.3. Initially, the columns were operated without bioaugmentation for 45 days, reaching steady-state effluent concentrations of 724  $\mu$ M for 2,4-DNT and 921  $\mu$ M for 2,6-DNT. Nitrite was not observed. On day 45, the columns were inoculated with 2,4-DNT and 2,6-DNT degrading mixed cultures. Effluent DNT concentrations decreased immediately, and nitrite production was observed. The pH decreased from 8.3 (influent) to 7.2–7.3 (effluent).

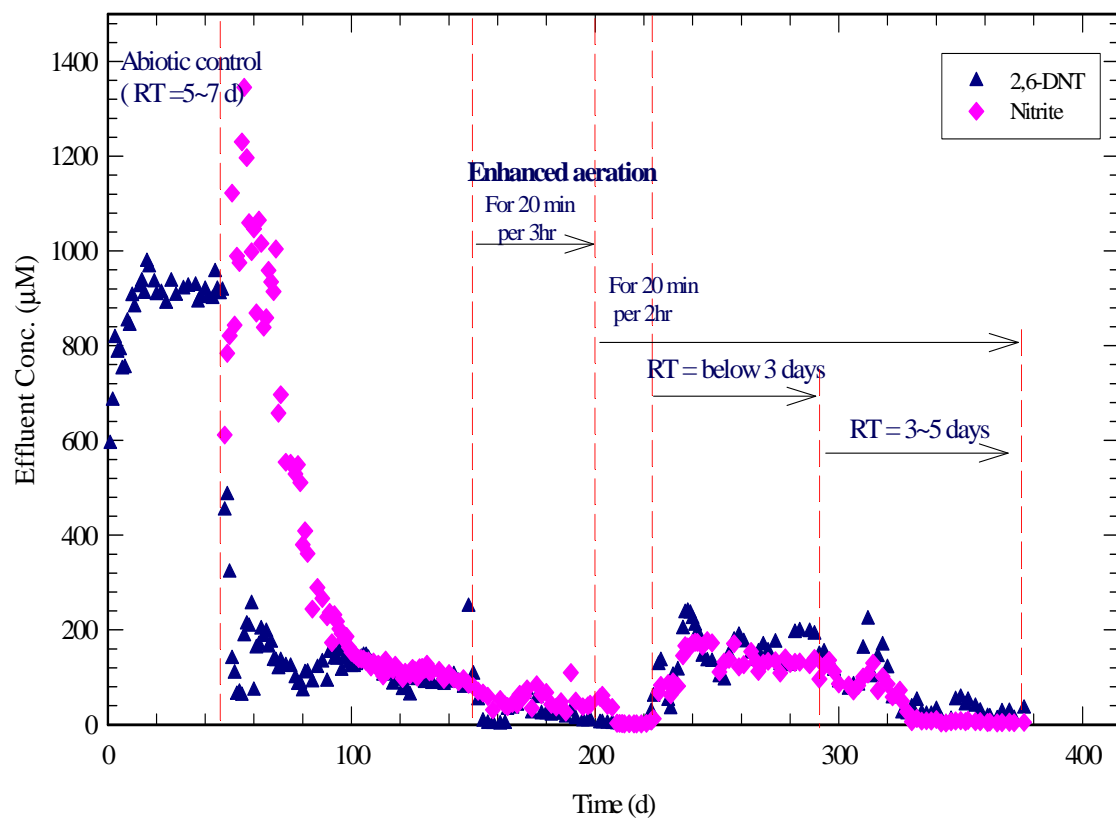
In Column 1 (Figure 4.2), effluent concentrations were below detection limits ( $0.1\ \mu\text{M}$ ) 13 d after augmentation. To evaluate the effect of infiltration rate on 2,4-DNT degradation rate, the RT was reduced from 5–7 d to 3–5 d at day 90. After 15 d at the higher flow rate, 2,4-DNT effluent concentrations increased to approximately  $30\ \mu\text{M}$ . On day 158, aeration was increased (20 min per 3 hr) to assess oxygen limitation. Initially the increase in aeration caused an increase of 2,4-DNT in effluent samples, followed by a return to low levels. Similar results were observed at more increased aeration (20 min per 2 hr). No observable increase in 2,4-DNT degradation was attributed to oxygen limitations.

In Column 2 (Figure 4.3), 2,4-DNT effluent concentration was  $90 \pm 8.6\ \mu\text{M}$  at steady state after augmentation, and nitrite production ( $103 \pm 9.2\ \mu\text{M}$ ) was observed. Slight increase of biodegradation (based on effluent 2,6-DNT concentrations) was caused by increase in aeration. On day 148, aeration capacity was increased (20 min aeration per 3 hr) where steady-state 2,6-DNT effluent concentrations decreased to  $24\ \mu\text{M}$ . More enhanced aeration (20 min/2 hr) did not influence 2,6-DNT effluent concentration. The effect of RT on 2,6-DNT degradation rate was evaluated, varying infiltration rate. When the RT was reduced from 5–7 d to  $< 3$  d at day 220, steady-state 2,6-DNT effluent concentrations increased to  $161 \pm 25\ \mu\text{M}$ , indicating decrease in 2,6-DNT degradation. When the RT was increased from  $< 3$  d to 3–5, immediate decrease of 2,6-DNT effluent was observed. As a result, observed increase of 2,6-DNT degradation was attributed to increase of RT.

Several conclusions can be drawn from the DNT effluent data. First, rapid degradation of both DNT isomers can occur at concentrations near saturation assuming



**Figure 4.2.** 2,4-DNT and  $\text{NO}_2^-$  in column effluents evaluating effects of system perturbations on degradation of 2,4-DNT in vadose zone (Column 1).



**Figure 4.3.** 2,6-DNT and  $\text{NO}_2^-$  in column effluents evaluating effects of system perturbations on degradation of 2,6-DNT in vadose zone (Column 2).

that oxygen and other nutrient requirements are available. This is particular and important finding with regard to 2,6-DNT, where it has been reported that 2,6-DNT cultures were inhibited at concentrations above 500  $\mu\text{M}$  (15). Second, infiltration rate will influence the extent of DNT biodegradation. DNT effluent concentration increased as RT decreased. 2,6-DNT biodegradation was more influenced by infiltration rate because the rate of 2,6-DNT biodegradation is typically slower than 2,4-DNT biodegradation (7). Third, while oxygen availability can be a major consideration in field site, it has a slight effect on DNT degradation under these experimental conditions. Similar observations regarding the minimal effect of enhanced aeration on 2,4-DNT degradation rate in historically contaminated soils have been reported by Fortner *et al.* (1).

#### **4.4.1.1. Nitrogen Balance.**

Nitrite production represents a quantifiable indicator for DNT degradation processes under aerobic conditions. The degradation of DNT isomers was evaluated on the basis of known stoichiometry (1.63 mol of nitrite release/1mol of DNT) (16). Nitrite production was observed in both columns after bioaugmentation (Figure 4.2 and 4.3) where non-stoichiometric nitrite release based on DNT loss was observed. A likely reason for the low levels of nitrite in the soil column was the oxidative metabolism of nitrite to nitrate. Nitrate effluent concentrations were monitored and results (Table 4.2) indicate that nitrogen was released near predicted stoichiometry (*i.e.*,  $\Sigma (\text{NO}_2^- \text{ and } \text{NO}_3^-)$  is similar to stoichiometric  $\text{NO}_2^-$  concentration based on DNT degraded). The presence of active nitrifying bacteria in the soil column occurred without bioaugmentation of



**Table 4.2.** Experimental nitrite and nitrate production, and comparison with predicted stoichiometry based on DNT removal (Column 1).

<b>Date (days)</b>	<b><math>\Delta</math> DNT (<math>\mu</math>M)</b>	<b>Exp. Nitrite (<math>\mu</math>M)</b>	<b>Eff. Nitrite (<math>\mu</math>M)</b>	<b>Exp. Nitrate (<math>\mu</math>M)</b>	<b>Eff. Nitrate (<math>\mu</math>M)</b>
98	1,039	1,694	401	1,292	1,177
101	1,052	1,714	303	1,411	1,220
104	1,052	1,715	287	1,427	1,305
108	1,078	1,757	207	1,549	1,380
117	1,078	1,757	233	1,524	1,457
134	1,063	1,733	81	1,652	1,625
144	1,076	1,755	61	1,693	1,666
153	1,080	1,760	99	1,661	1,672
156	1,073	1,748	87	1,662	1,669
158	1,067	1,740	97	1,642	1,595
159	1,091	1,779	191	1,587	1,352
164	992	1,616	542	1,074	1,274
168	1,039	1,694	426	1,268	1,488
187	1,055	1,720	119	1,601	1,740
190	1,057	1,722	118	1,605	1,693
192	1,055	1,720	118	1,602	1,688
195	1,082	1,764	96	1,668	1,762
197	1,077	1,755	237	1,518	1,798
201	1,098	1,790	86	1,703	1,494

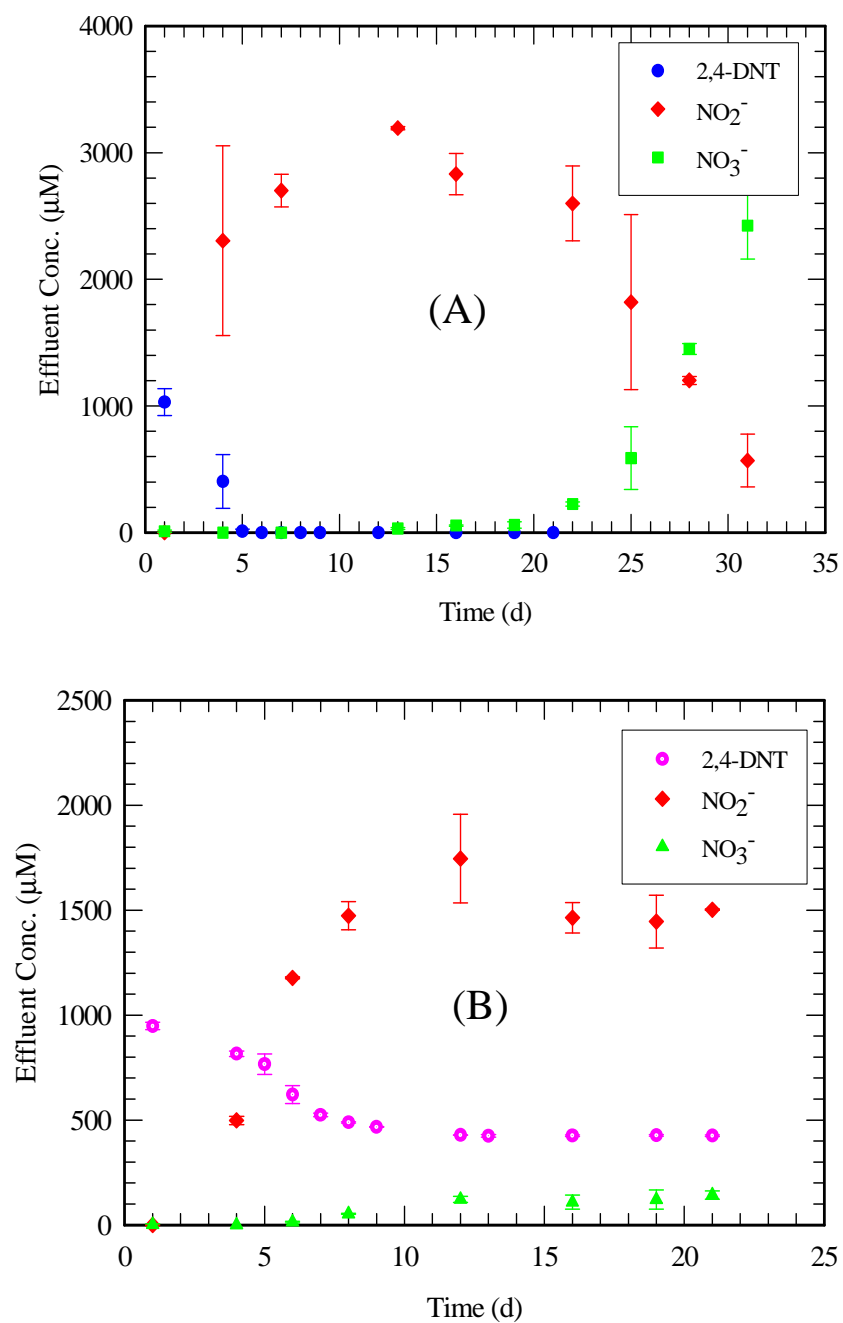
(Eff.): experimental effluent concentration,  
 (Exp.): stoichiometrically expected concentration

nitrifiers. Nitrate production was corresponded with amounts of nitrite degraded (*i.e.*, 1 mM nitrite is converted to 1 mM nitrate (9)) and total nitrogen was present in the reactor as stoichiometric quantities for influent 2,4-DNT concentration.

#### ***4.4.2. Shake Flask Experiments: Screening Activity of Nitrifying Bacteria.***

To confirm the presence of nitrite oxidizers in the column system, shake flask studies were conducted with soil samples from Column 1 and mineral media spiked with 2,4-DNT. Results (Figure 4.4A) present 2,4-DNT degradation along with the profiles of nitrite and nitrate concentration. Complete degradation of 2,4-DNT was observed in shake flasks after 5 d incubation. Correspondingly, nitrite as indicator was produced. On day 22, nitrite began to decrease corresponding to increase of nitrate, confirming the presence of an active and transferable nitrifying culture. It is speculated that nitrite oxidizers present in the soils are responsible for the oxidation of nitrite to nitrate (nitrification), explaining the difference in experimentally observed vs. expected nitrite based on the DNT removal in the column. Additionally, 2,4-DNT did not inhibit nitrification. Complete degradation of nitrite occurred in the presence of saturated 2,4-DNT (1000  $\mu$ M). This indicates that no observed inhibition of nitrification was attributed to the presence of 2,4-DNT under appropriate pH condition. However, 2,4-DNT may influence nitrification indirectly because 2,4-DNT degradation causes the pH drop.

Another shake flask was established with the mixed cultures to evaluate the influence of low pH (a pH of 6) on nitrification. Over 21 day incubation, no significant nitrite decrease and a neglecting production of nitrate were observed as the evidence of low pH inhibition on nitrifying bacteria (Figure 4.4B). Since 2,4-DNT was slightly



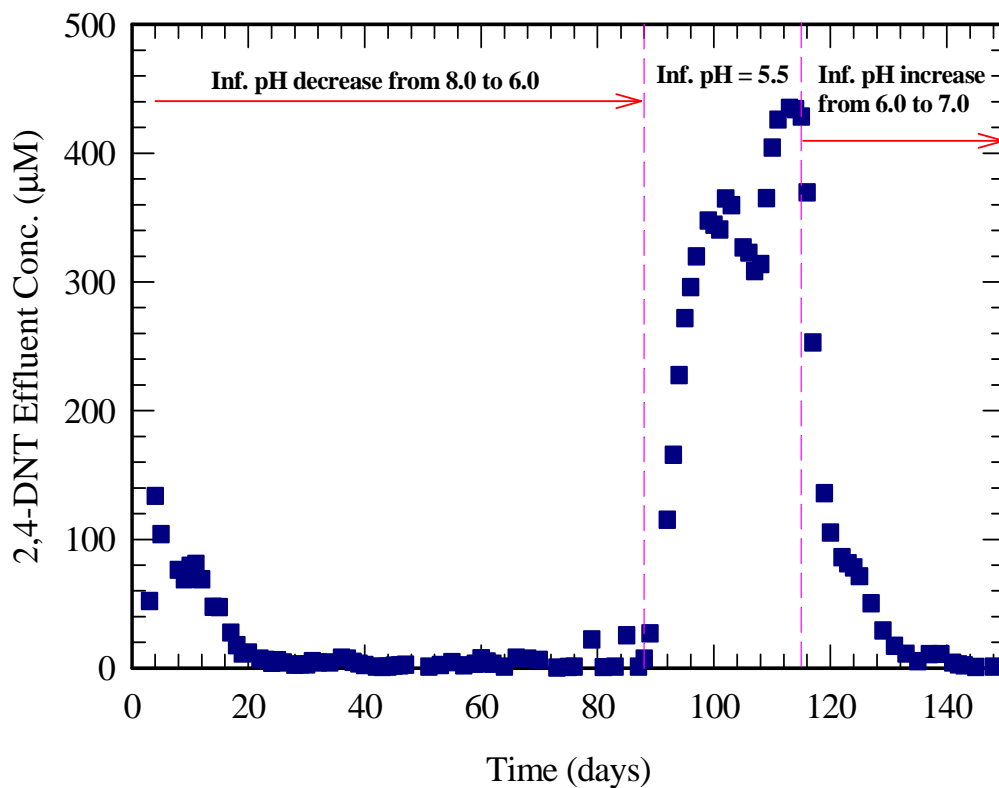
**Figure 4.4.** Shake flask test with soil samples from Column 1: Initial the value of (A) pH 8 and (B) pH 6 in reactor.

consumed, it is clear that low pH inhibited both nitrification and DNT degradation process.

#### ***4.4.3. Columns Studies with Influent pH as a Variable.***

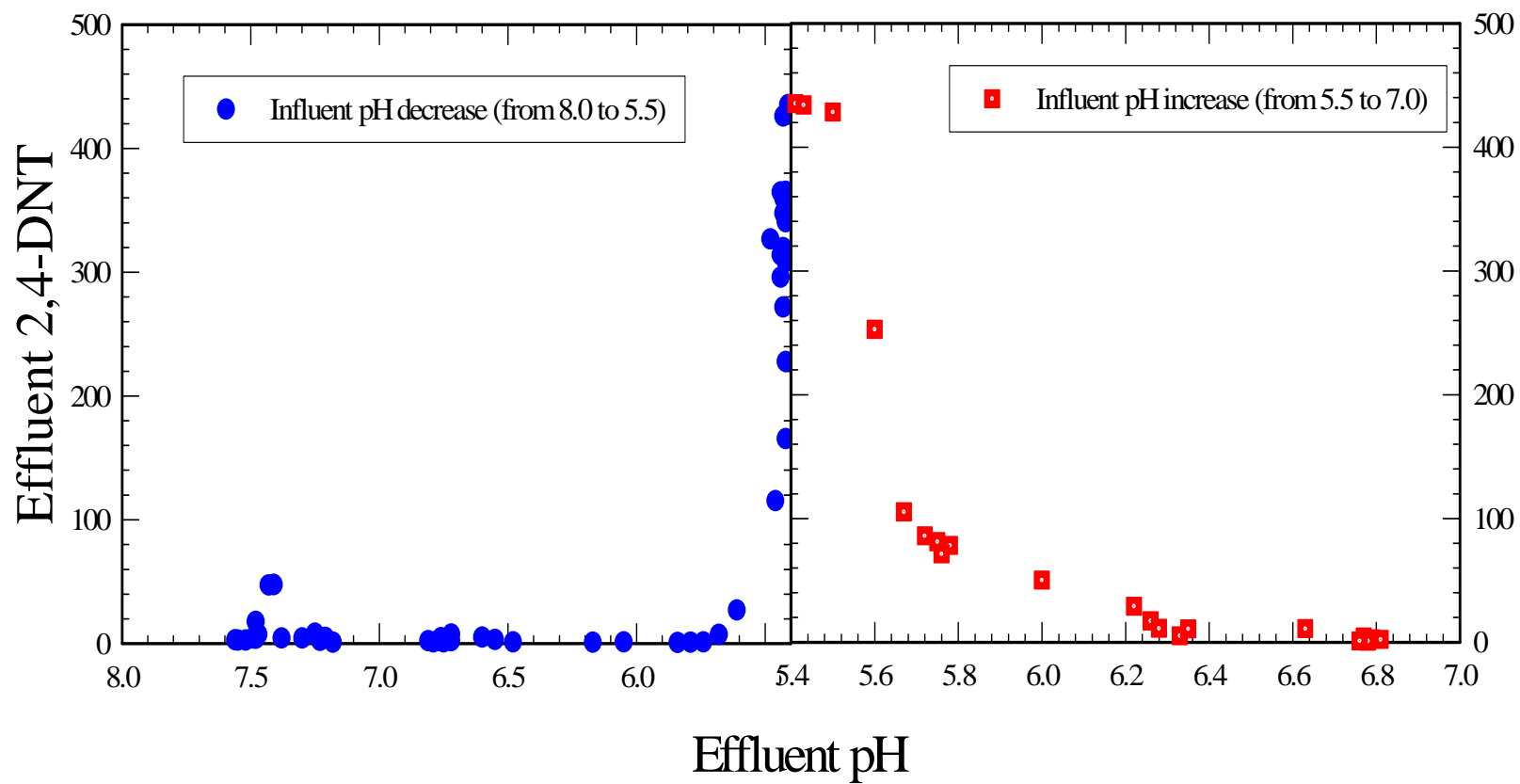
Column studies, referred to as Columns 3 and 4 for 2,4-DNT and 2,6-DNT respectively, were conducted to estimate threshold pH which inhibits DNT biodegradation in a simulated vadose zone. Results for Column 3 are presented in Figure 4.5 where table lists influent pH over time. In this system the decrease of influent pH (from 8.0 to 6.0) did not influence biodegradation of 2,4-DNT, but inhibition of degradation was observed at influent pH=5.5. 2,4-DNT effluent concentration increased rapidly at pH value of 5.5. The influent pH was then raised to monitor the rate of recovery as measured by effluent 2,4-DNT. When the influent pH increased from 5.5 to 6.0, 2,4-DNT degradation recovered rapidly (from 435  $\mu\text{M}$  to 86  $\mu\text{M}$ ), and medium infiltration with influent pH values of 6.5 and above supported the initial extents of biodegradation at influent pH=8.0.

Effluent pH can be used to assess the effect of soil pH on 2,4-DNT degradation. 2,4-DNT effluent concentrations vs. effluent pH were presented in Figure 4.6. At a influent pH of 8.0, effluent pH was dropped to 7.12 and steady-state 2,4-DNT effluent concentration was 5.2  $\mu\text{M}$ . At effluent pH ranging from 7.6 to 5.7, 2,4-DNT effluent concentration stabilized at trace level, indicating sustained degradation of 2,4-DNT. Low pH in feed solutions caused corresponding decrease of effluent pH. When effluent pH decreased to 5.4 (influent pH was 5.5), rapid increase of 2,4-DNT effluent concentration was observed. Subsequently, when effluent pH increased from 5.4 to 5.8, 2,4-DNT



Column 3 Running Time (days)	Influent pH
1	8
28	7.5
42	7
57	6.5
73	6
85	5.5
113	6
123	6.5
135	7

**Figure 4.5.** Soil column studies studying pH effect: 2,4-DNT degradation according to influent pH time line (Column 3).



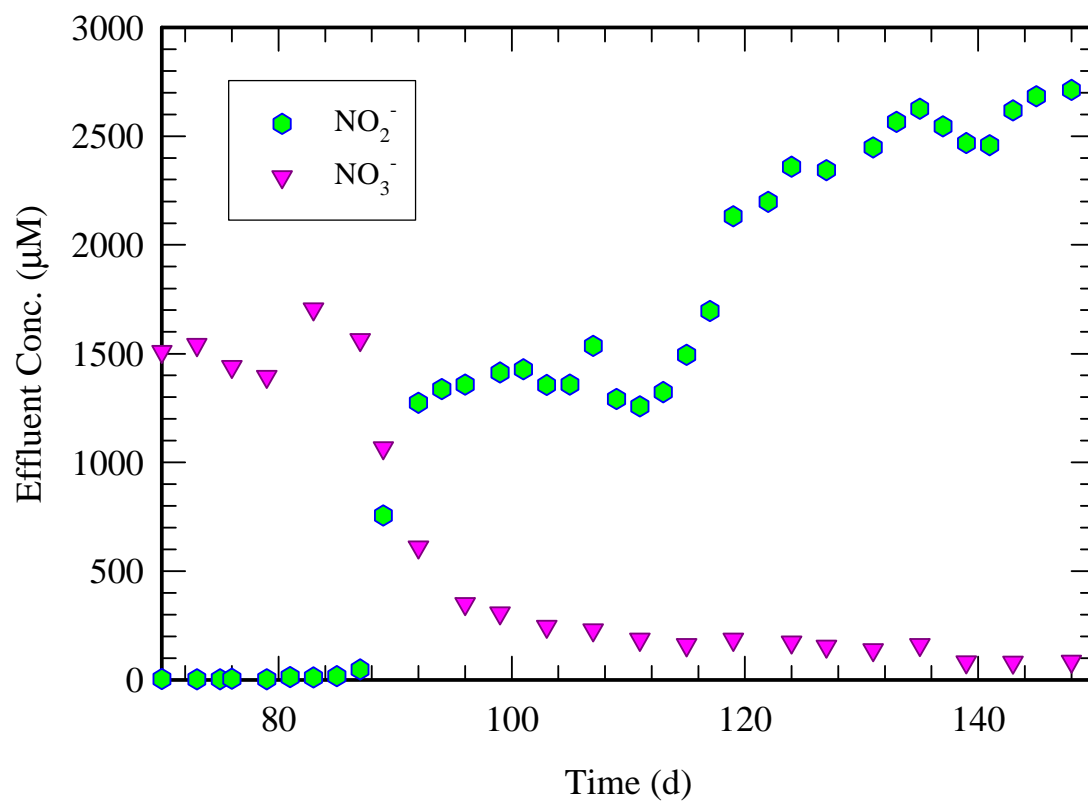
**Figure 4.6.** The correlation between effluent pH and effluent 2,4-DNT in Column 3. Initial influent pH values were decreased from 8.0 to 5.5 (Left) and the pH was then raised in a similar stepwise manner (Right).

effluent concentration decreased rapidly to low level and reached near detection limits at effluent pH value of 6.8, indicating complete recovery of 2,4-DNT degradation.

Nitrite and nitrate analyses were conducted in Column 3 (Figure 4.7). The nitrite level was near detection limits ( $0.08\ \mu\text{M}$ ) at influent pH ranging from 8.0 to 6.0, but the levels of effluent nitrite began to increase when the influent pH was 5.5. At steady state the nitrite concentration was  $1,400\ \mu\text{M}$ . Even though the values of influent pH increased to 7.0, nitrite was continuously produced and elevated to  $2,700\ \mu\text{M}$ . As expected with increasing nitrite was a commensurate drop in the level of nitrate in effluent sample. Initial nitrate effluent concentration was approximately  $1,500\ \mu\text{M}$ . Effluent nitrate began to decrease by decrease of influent pH to 5.5 and stabilized at trace level. This observation indicates nitrification was inhibited by low pH, and did recover after the pH increase.

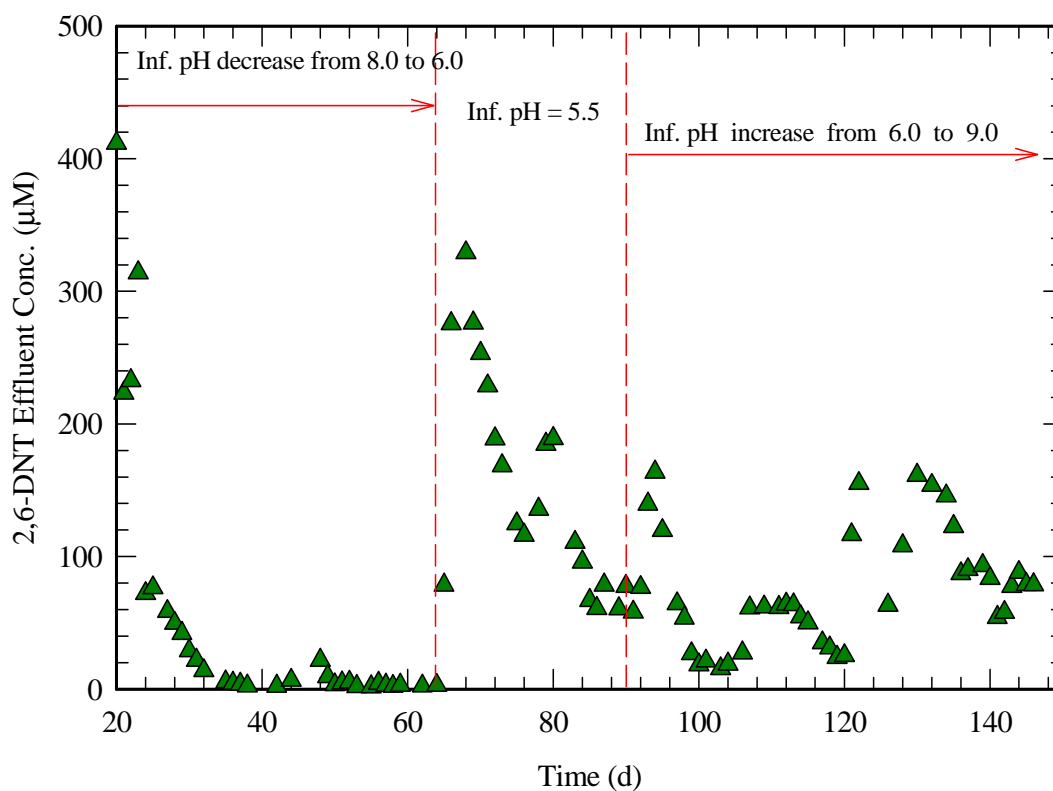
Column 4 was employed to investigate the impact of pH on 2,6-DNT biodegradation. As presented in Figure 4.8, influent pH ranging from 8.0 to 6.0 did not influence 2,6-DNT degradation, while a rapid increase of 2,6-DNT effluent concentration was observed at influent pH of 5.5. On day 90, influent pH increased to 6.0 in an attempt to recover 2,6-DNT degradation, but no significant reduction of 2,6-DNT effluent concentrations were observed. When the influent pH was increased from 6.0 to 9.0 (7.0, 8.0, and 9.0 on day 103, 120, and 132, respectively), 2,6-DNT effluent concentrations were still higher than those before pH drop (Figure 4.8). At influent pH values of 8.0 to 9.0, the effluent pH increased to above 7.0, but the 2,6-DNT effluent concentrations never returned to level observed prior to the reduction of pH.

In summary, 2,4-DNT and 2,6-DNT degradation in both systems were inhibited at



**Figure 4.7.** Profiles of nitrite and nitrate produced in Column 3.





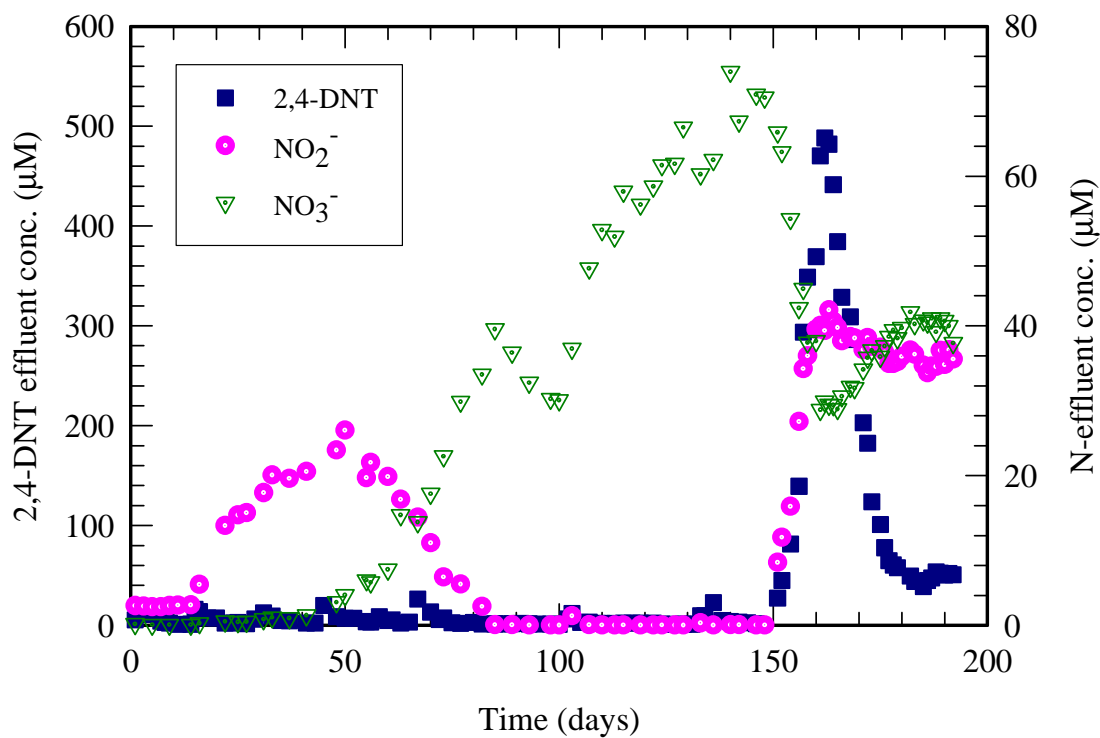
Column 4 Running Time (days)	Influent pH
1	8
23	7
48	6
64	5.5
90	6
103	7
120	8
132	9

**Figure 4.8.** Soil column studies studying pH effect: 2,6-DNT degradation according to influent pH time line (Column 4).

pH values approaching 5.5 and below. This has implications in vadose zones underneath highly contaminated soils undergoing bioremediation or rainwater infiltration. If degradation in the soil is rapid, low pH waters will enter the vadose zone, carrying DNT, and natural attenuation may not occur. The natural buffering capacity of the subsurface may become an important site variable. While the pH effect could be reversed for 2,4-DNT degradation systems by raising the influent pH, the 2,6-DNT recovery was slow and erratic, suggesting the need to carefully control pH to sustain 2,6-DNT degradation. Additionally low pH (5.5) caused nitrite elevation, as expected due to a loss of nitrification activity. Active nitrification can reduce nitrite inhibition of DNT degradation, thus pH effects may be coupled with nitrite toxicity.

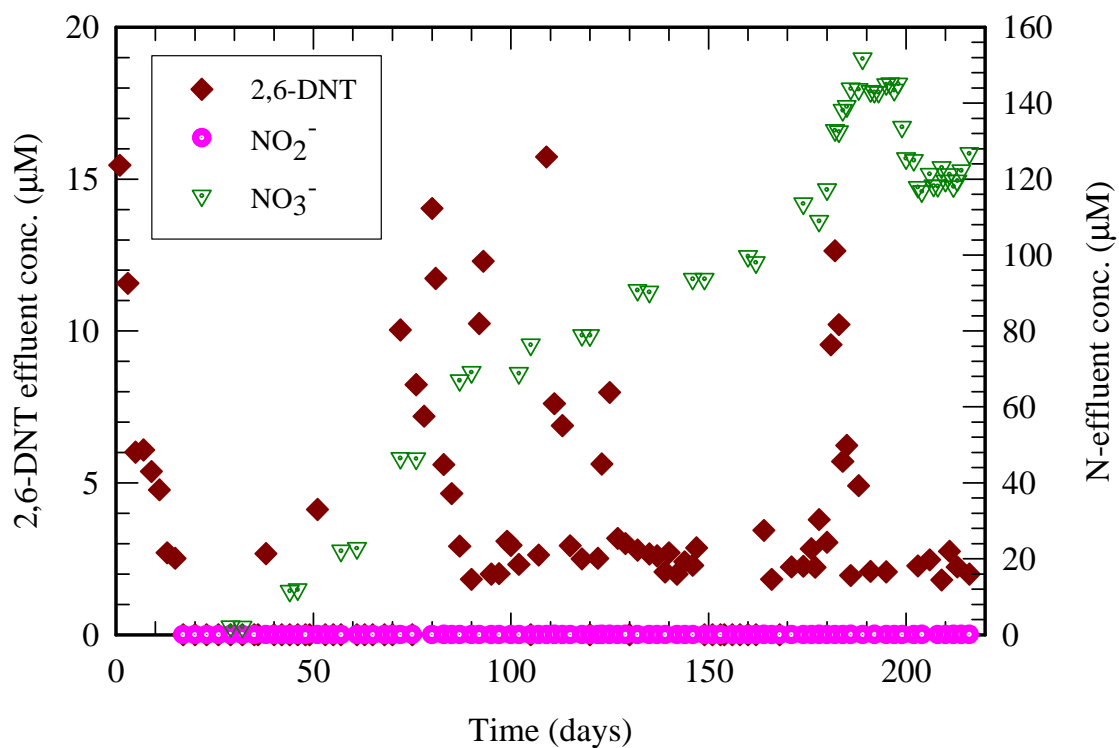
#### **4.4.4. Columns Studies with $\text{NO}_2^-$ Variable in the Influent.**

Column studies were conducted to assess effects of nitrite on DNT degradation in the simulated vadose zone soil columns (Column 5 and 6 for 2,4-DNT and 2,6-DNT, respectively). Tables in Figure 4.9 and 4.10 present the influent nitrite concentration in the feed solution over the operation time. Effluent profiles of 2,4-DNT in Column 5, along with  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  concentration, is presented in Figure 4.9. In the Column 5, initial influent nitrite (15 mM) caused perturbed elevation of nitrite effluents without varying of 2,4-DNT degradation. After initial addition of nitrite, the influent nitrite concentration increased from 20 to 65 mM with interval of 5 or 10 mM. During 112 day after addition of 20 mM  $\text{NO}_2^-$ , nitrite effluent concentration sustained near 0.11 mM, while nitrate effluent concentration increased to 74 mM of  $\text{NO}_3^-$ . 2,4-DNT effluent concentration stabilized at low level (approximately 2.25  $\mu\text{M}$ ) during this period. On day



Column 5 Running Time (days)	Influent Nitrite Conc. (mM)
15	15
29	20
45	25
58	30
72	35
87	40
102	50
117	60
131	65
144	70

**Figure 4.9.** Soil column studies studying NO<sub>2</sub><sup>-</sup> effect: Variation of 2,4-DNT, NO<sub>2</sub><sup>-</sup>, and NO<sub>3</sub><sup>-</sup> effluents according to influent NO<sub>2</sub><sup>-</sup> time line (Column 5).



Column 6 Running Time (days)	Influent Nitrite Conc. (mM)
35	10
48	20
63	40
78	60
92	70
107	80
122	90
137	95
151	100
164	110
181	120 ~ 130

**Figure 4.10.** Soil column studies studying  $\text{NO}_2^-$  effect: Variation of 2,6-DNT,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  effluents according to influent  $\text{NO}_2^-$  time line (Column 6).

144, nitrite concentration in feed solutions was 70 mM where rapid elevation of 2,4-DNT effluent concentration was observed. Commensurate with loss of 2,4-DNT degradation, nitrite effluent concentrations increased to 35 mM, paralleling the decrease of nitrate to 40 mM. There was no effort to identify the reasons for the sudden elevation of nitrite and reduction of nitrate, but clearly the presence of nitrifying cultures can decrease the nitrite toxicity for 2,4-DNT degraders.

Data presented in Figure 4.10 represent 2,6-DNT, nitrite, and nitrate concentrations in effluents for Column 6. Nitrite concentration in feed solutions ranged from 10 to 130 mM for this system. In comparison to Column 5, there was no observable increase of nitrite concentration despite addition of higher influent nitrite concentration. Immediate and continued nitrate evolution was observed after addition of external nitrite. Over the entire operation, 2,6-DNT effluent concentration maintained at relatively low concentration ( $< 20 \mu\text{M}$ , often below 5). This indicates that 2,6-DNT degrading mixed cultures were less sensitive for nitrite than 2,4-DNT degrading mixed cultures, and the presence of nitrite oxidizer in the soil can make DNT degradation activity occur at a faster rate.

#### **4.5. Conclusions**

The biodegradation of both DNT isomers was observed in simulated vadose zone column studies which were bioaugmented and provided DNT concentrations near saturation. This suggests that 2,4-DNT and 2,6-DNT degradation can be expected in underlying DNT contaminated soils when oxygen and other nutrient requirements are available. The results from the soil column study demonstrated that nitrite will not

always be a quantitative indicator of DNT degradation. In fact, nitrite concentrations may become below detection limits, creating a false negative analysis for DNT degradation outside of areas of heavy soil contamination. Also, biological nitrification processes converting nitrite to nitrate could decrease exposure of DNT degraders to nitrite, resulting in reduction of nitrite toxicity. Factors controlling DNT mineralization in vadose zone through column studies were characterized and the pH strongly inhibited DNT (2,4-DNT and 2,6-DNT) biodegradation below a pH of 6.0. Nitrite oxidizer was also inhibited by amendment of low pH. This demonstrates that pH drop from DNT biodegradation may have effects on the reduction of bioactivity of both DNT degraders and nitrite oxidizers. Conclusively, effective control of pH (*e.g.*, buffer addition) is needed for viable *in situ* bioremediation of DNT.

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## CHAPTER 5

### **Pilot Scale Systems: DNT Bioremediation Coupled with Denitrification Processes**

#### **5.1. Overview**

To gain further insight on potential of *in situ* bioremediation strategies, pilot scale systems were constructed to test the treatability of a DNT contaminated soil (collected from Barksdale, WI) through water infiltration. Soils obtained were contaminated with both 2,4-DNT and 2,6-DNT. Two identical pilot systems (termed DNT BioPilot 1 and 2) were operated, each with three reactors in series: a soil tank for DNT contaminated soils; a denitrification (DNR) tank for sequential denitrifying step; and a recycle tank for recirculation. No additions (*e.g.*, nutrients and buffer solutions) other than biodegradable organic addition for active denitrification in DNR tank were employed in these studies, and DNT, nitrite, nitrate, and COD were monitored from the outlet of each tank.

A primary objective was the evaluation of rate and extent of DNT degradation in field soils under simulated field conditions during water infiltration. The level of 2,4-DNT effluent was reduced below detection limit (0.02 mg/L) as soon as the integrated system (DNT biodegradation and denitrification process) was started. 2,6-DNT was observed in soil effluent at day 1 of operation, equilibrating in concentration at approximately 33 days, then dropped to near detection limits after 90 days of operation. From the start of operation, nitrite and nitrate were observed in the effluent of the soil tank, and the amounts increased with increased DNT degradation. Soil effluent also contained recalcitrant nitroaromatics (*i.e.*, dinitroxylenes and trinitrotoluene as identified

by GC-MS) and unidentifiable organics which remained present even in extended microcosm studies.

Nitrite was evolved with DNT depletion, and nitrate (oxidized from nitrite via nitrification) was present in the effluent from the soil tanks even when DNT was not detected in the effluent. An anaerobic denitrification system was used to remove nitrite and nitrate evolved from DNT biodegradation. Within the denitrifying step, nitrite and nitrate were decreased ( $< 1$  mg/L) when provided biodegradable COD (as dextrose). Interestingly, excess additions of COD decreased 2,6-DNT degradation during recycle. As a result, COD/N ratio is an important control variable for *in situ* bioremediation to control DNT, nitrate, and nitrite when recycle is employed.

## **5.2. Introduction**

Naturally occurring aerobic bacteria that are able to metabolize DNT as their sole source of energy have been identified at the Barksdale site (Chapter 3). Previous work on soils from this site has shown that DNT biodegradation occurs under appropriate conditions (Chapter 3). Also, biodegradation within the vadose zone can be sufficient to attenuate DNT leaching from surface soils before reaching the water table (Chapter 4). These observations serve to increase the potential for *in situ* bioremediation or natural attenuation (NA) strategy.

Interestingly, successful bioremediation requires more than just DNT removal. Nitrite is a product of DNT transformation and it too must be treated, as it is a regulated pollutant of water (1-4). Nitrite evolution associated with DNT depletion may display recalcitrance in aerobic environments (5, 6), in particular when associated with high DNT

levels. Nitrite can be converted to nitrate by nitrifying bacteria under aerobic conditions (Chapter 3 and 4) and thus both nitrite and nitrate may be formed as a result of DNT degradation. Because of the potential toxicity of nitrite and nitrate (7-10), the treatment of both to low concentrations is required. Redox conditions in the vadose zone can be an important consideration in the fate of nitrite and nitrate. Oxygen in soil gas can be completely depleted in vadose zone. Denitification processes under anaerobic conditions cause the reduction of nitrogen (*i.e.*, nitrite and nitrate) as electron acceptors for microbial growth (9, 11-13).

This study examined the feasibility of *in situ* bioremediation for the removal of 2,4-DNT, 2,6-DNT, nitrite, and nitrate in a two stage pilot scale system. The experiment was conducted with contaminated soil obtained from a historically contaminated field site (Barksdale, WI), and the study evaluated the extent of DNT degradation during infiltration of water. Subsequently water passed into a denitification system for  $\text{NO}_2^-$ / $\text{NO}_3^-$  removal. Results demonstrate the degradation of 2,4-DNT and 2,6-DNT biodegraded in this soil pilot system under conditions that simulate water infiltration and control of evolved nitrogen achieved through the augmentation of degradable COD.

### **5.3. Materials and Methods**

#### **5.3.1. Chemicals.**

Methanol and dextrose were obtained from Fisher Scientific and Sigma-Aldrich, respectively.  $\text{KNO}_3$  (Fisher Scientific) was used as nitrogen source for denitrification cultures. Colorimetric reagents for Chemical Oxygen Demand (COD) measurement were

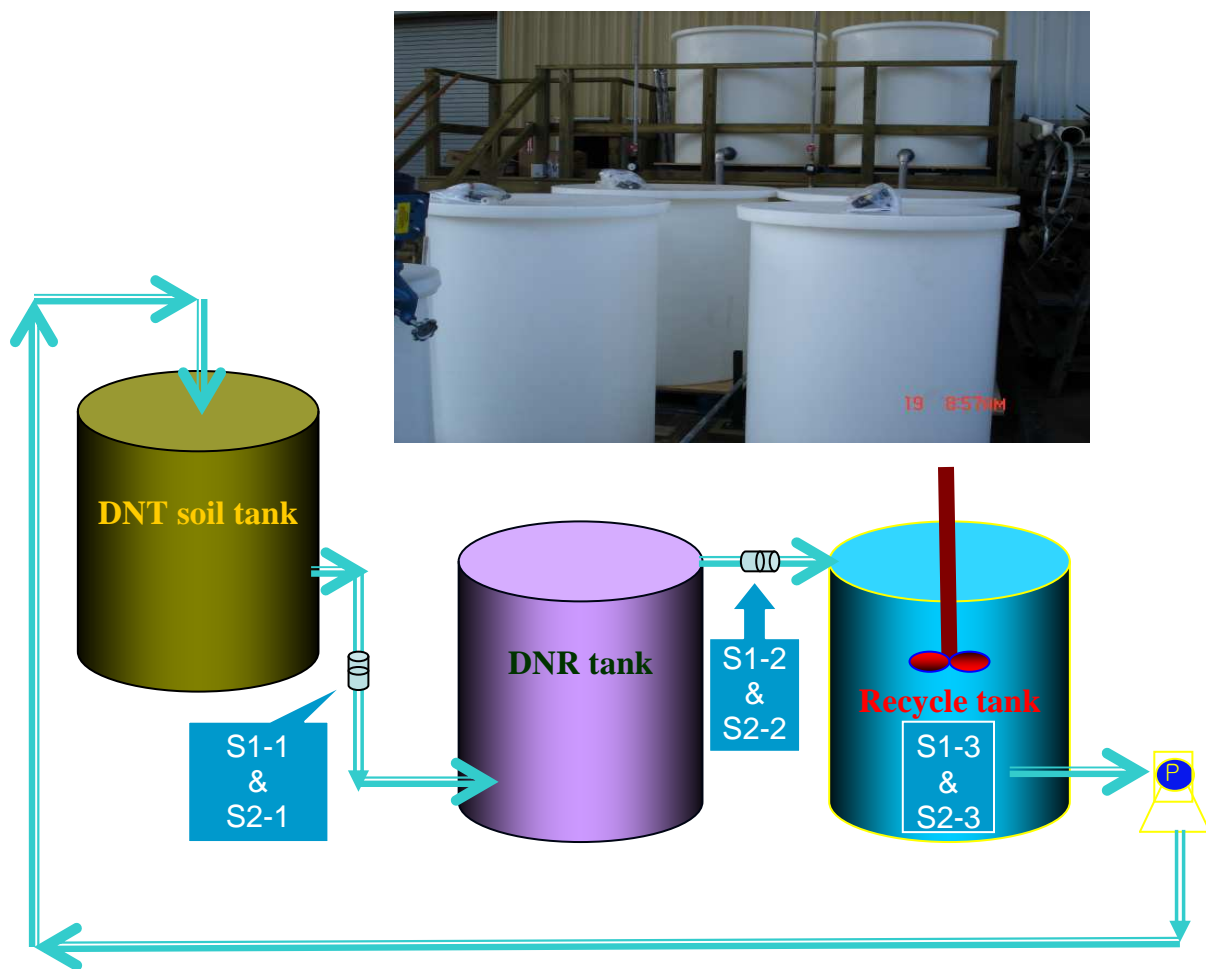
purchased from HACH Corporation. Other chemicals, analytical standards, and organic solvents were described in Chapter 3.

### **5.3.2. Pilot Studies**

#### **5.3.2.1. Apparatus and Operation.**

Two pilot scale biodegradation systems (termed DNT BioPilot) were constructed in parallel (Figure 5.1), each consisting of three vessels: a soil reactor, a denitrification (DNR) reactor, and a recycle reactor. Each soil tank was a 375 gallon polyethylene tank, equipped with water level sensors to prevent overflow. Soil tanks were first filled with porous support media to prevent washout of contaminated soil. The support media was comprised of three layers starting with pea gravel (6 inch layer) at the bottom, followed by a 6 inch layer of 3.3 mm (diameter) sand, and finally another 6 inch layer of 1.1 mm (diameter) sand. Subsequently, the contaminated soil loaded with contaminated soils (628 kg in soil tank 1 and 618 kg in soil tank 2). The stratified layers of contaminated soil and support sand placed at the bottom of the soil tank were described in Table 5.1. Subsequently, a 375 gallon polyethylene DNR tank with large gravel media (granite) was connected at the lower platform, followed by a 200 gallon polyethylene recycling tank, which was mechanically mixed.

Intermittent water flow from the recycle tank to the soil tank was conducted by means of booster pumps and an automated solenoid control system. External carbon addition to the denitrification system was carried out by a pulse pump into the bottom of DNR tank or direct addition to the top of the system. There was no acid/base addition, or external pH control.



**Figure 5.1.** Schematics of pilot scale biodegradation systems (DNT BioPilot).

**Table 5.1.** The soil matrix and depth of layers in the soil tank.

<b>Matrix</b>	<b>Depth of layer</b>
Contaminated Soil (top layer)	18 inch
1.1 mm (diameter) Sand	6 inch
3.3 mm (diameter) Sand	6 inch
Pea Gravel (bottom layer)	6 inch

#### **5.3.2.2. Enrichment of Denitrifying Cultures.**

Microcosms were operated in closed batch systems, constructed with serum bottles (160 mL, Wheaton Co. Millville, NJ) containing 10 g sand and minimal medium for denitrifying bacteria (80 mL) (14), sealed with rubber stoppers and aluminum crimp seals (Wheaton Co. Millville, NJ), and sparged with N<sub>2</sub> gas to remove O<sub>2</sub>. To assess the transformation of nitrite, supernatants from microcosms were transferred to soil free media bottle (500 mL) with a cap and ports containing fresh media and nitrite (100 µM) after several days. The pattern of COD/N ratio (*i.e.*, COD consumption as a function of the N removed) and nitrogen profiles were assayed to confirm denitrification activity (15).

To investigate the effect of DNT presence on denitrification rate and extent, DNT and nitrite amended microcosms were established with denitrifying cultures, with a DNT free amended microcosm as a control. Aliquots of the denitrifying culture (10 mL) were added to serum bottles (160 mL) containing DNT/nitrite or only nitrite amended media (90 mL). Experiments were conducted with various initial DNT concentrations (0 to near saturation). All microcosms were incubated at 20°C on rotary shaker.

#### **5.3.2.3. Baseline Estimation.**

An initial study was conducted in the pilot system to determine concentrations of DNT and nitrite that would leach from the soil at startup. Water (100 Gal/day for 6 days) was recycled through the each system, and samples were taken daily from three sampling points (outlets from soil tank, DNR tank, and recycle tank).

#### **5.3.2.4. Active DNR Tanks Construction.**

Enriched denitrifying cultures were amended with dextrose (as additional carbon source),  $\text{KNO}_3$  (as nitrogen source), and mineral media into DNR tanks. The cultures were enriched for 24 days before system integration to establish viable DNR tanks. A COD: $\text{NO}_3^-$ -N ratio of 8:1 was employed for initial DNR startup to avoid carbon limiting effects, as suggested in the literature (16, 17). The COD,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  were measured to monitor activity of denitrification.

#### **5.3.2.5. Integrated BioPilot Operation.**

After preliminary baseline studies and inoculation/stabilization of DNR tank, an integrated DNT mineralization-denitrification system were operated to treat historically DNT contaminated soil and to control corresponding  $\text{NO}_2^-$  and  $\text{NO}_3^-$ . The influent loading rate to both soil tanks was initially was 20 Gal/day. On day 24 the loading to system1 was changed from 20 Gal/day to 10 Gal/day, but was constant for System 2 at 20 Gal/day. Denitrification was enriched by the addition of varying the amount of methanol, and then later dextrose added (System 1, day 45 methanol, day 68 dextrose; System 2, day 36 methanol, day 59 dextrose). Finally, as an attempt to remove excess COD, effluent was allowed to stand in the recycle tank (as a batch reactor), which was mechanically stirred, for 4 months (120 days) upon the completion of the integrated system (100 days).

#### **5.3.3. Sample Collection and Preparation.**



Samples were taken daily from three sampling points for each system (duplicate systems): (1) soil tank outlet (2) denitrification (DNR) tank outlet and (3) recycle tank outlet. Each sample (200 to 300 mL) was transferred to borosilicate vials after direct measurement of pH (Denver Instrument pH 220), and stored at 4°C for later analyses. All aqueous samples taken in during study were filtered through a PTFE filter unit (0.22 µm, Millipore) prior to analyses.

#### **5.3.4. *Experimental Analysis***

##### **5.3.4.1 High Pressure Liquid Chromatograph (HPLC) Analysis.**

Separation and quantification of DNT was accomplished primarily via HPLC (Agilent, 1100 series) equipped with a Zorbax® SB-C18 (18) or a Hypercarb® porous graphite column (5) and a diode array detector. Analytical protocols were described in Chapter 3.

##### **5.3.4.2. Nitrite and Nitrate Measurements.**

Dissolved nitrite and nitrate was measured colorimetrically or by Dionex IC following standard methods for Water and Wastewater Treatment method 4500-NO<sub>2</sub><sup>-</sup> or 4500-NO<sub>3</sub><sup>-</sup> (19).

##### **5.3.4.3. COD Analysis.**

The measurement of COD was conducted using a colorimetric protocol developed by the HACH Corporation. For sample preparation, filtered aqueous samples (2 mL) were put into HACH COD vials containing color reagents (3 mL) and oxidants, and were

hand-tumbled for several times over a sink to mix. The prepared vials were placed on COD reactor (DRB200, HACH) preheated to 150°C, and incubated for 2 hr, then allowed to cool at room temperature, whereby samples were measured at 620 nm (Cary 50, Varian Inc.).

#### **5.3.4.4. Gas Chromatograph (GC) Analysis.**

GC analysis was conducted to confirm data quality and consistency (20). A liquid-liquid extraction procedure was used for sample preparation: Hexane (1 mL) was added to liquid sample (1 mL) in a standard sample vial (3 mL). The vial was mixed, and allowed to separate (*i.e.*, hexane overlaying the water layer). A portion of the hexane layer was withdrawn and analyzed on a Hewlett Packard (HP) 6890 GC with a mass selective detector (MS) equipped with a HP-5MS 5 % phenyl methyl siloxane capillary column (30 m×0.25 mm ID×0.25 µm film). The GC was used in a splitless mode with an injector temperature 250°C. The oven temperature program increased at 10°C /min linear ramp: from 80°C to 190°C.

#### **5.3.5. *Soil Extraction Procedure.***

Representative soil samples from each soil reactor were transferred to small glass jars, and stored at 4°C prior to extraction and analyses. Four sub samples (approximately 0.05 g each) were extracted for each homogenized soil. The experimental protocols were described in Chapter 3 (5, 21).

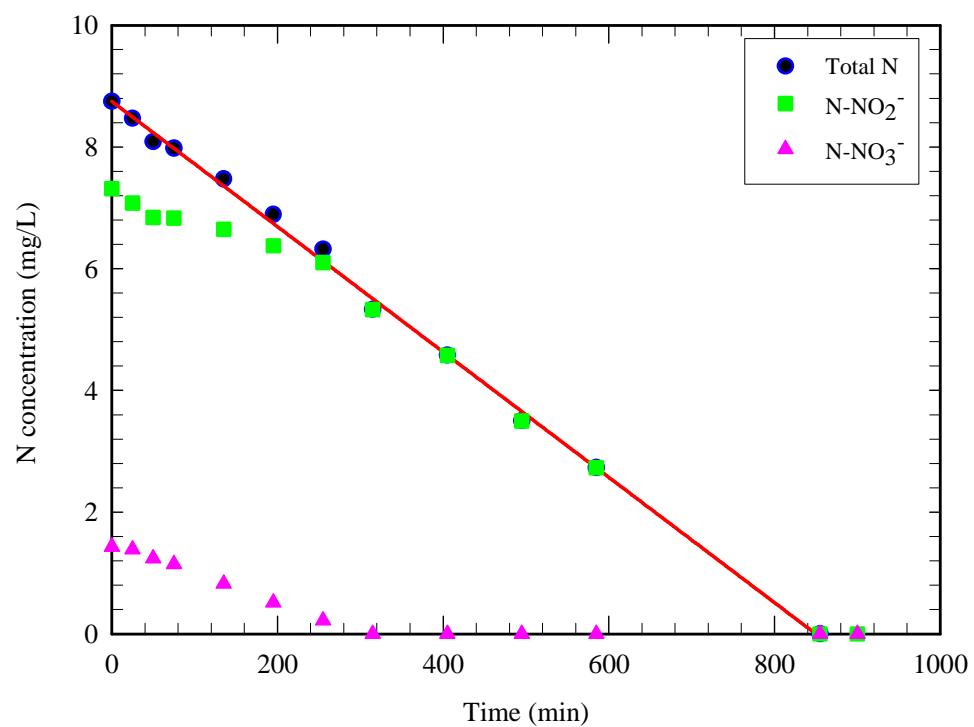
## 5.4. Results and Discussion

### 5.4.1. Preliminary Denitrification Studies.

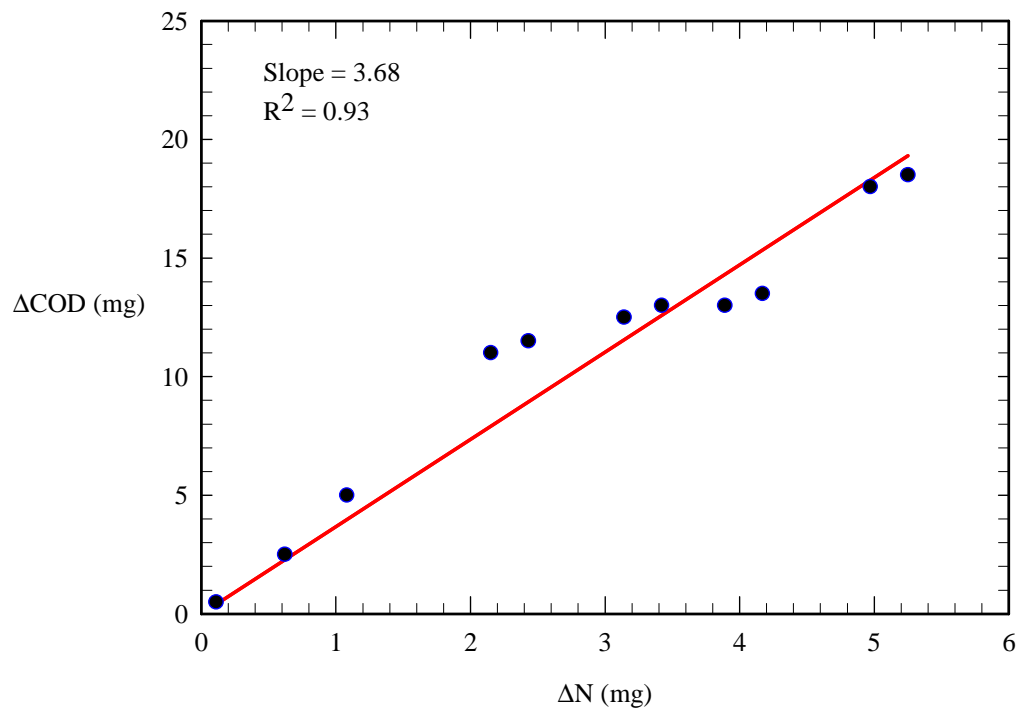
Preliminary denitrification tests were conducted in separate batch reactors, operated under anoxic conditions. The total nitrogen concentration was 8.75 mg/L (7.32 mg N-NO<sub>2</sub>/L + 1.43 mg N-NO<sub>3</sub>/L) and excess dextrose (as COD) was provided to maintain anoxic conditions. The nitrogen profiles (vs. time) are presented in Figure 5.2. Nitrogen was completely depleted and a linear trend of total nitrogen vs. time was shown during denitrification tests. These cultures were later used for inoculation of DNR tanks.

Critical to DNR design is the amount of substrate necessary for efficient denitrification (*i.e.*, COD: N ratio). Methanol is an effective substrate for this purpose. The amount of COD removed ( $\Delta$  COD) was plotted against the amount of nitrate nitrogen reduced ( $\Delta$  N-NO<sub>3</sub>) in the same time interval. A linear correlation was observed (Figure 5.3) and 3.68 mg COD/ mg N-NO<sub>3</sub><sup>-</sup> was obtained as the appropriate ratio of COD used for this particular culture. A similar value (3.71) has been reported by Rittman and McCarty (22).

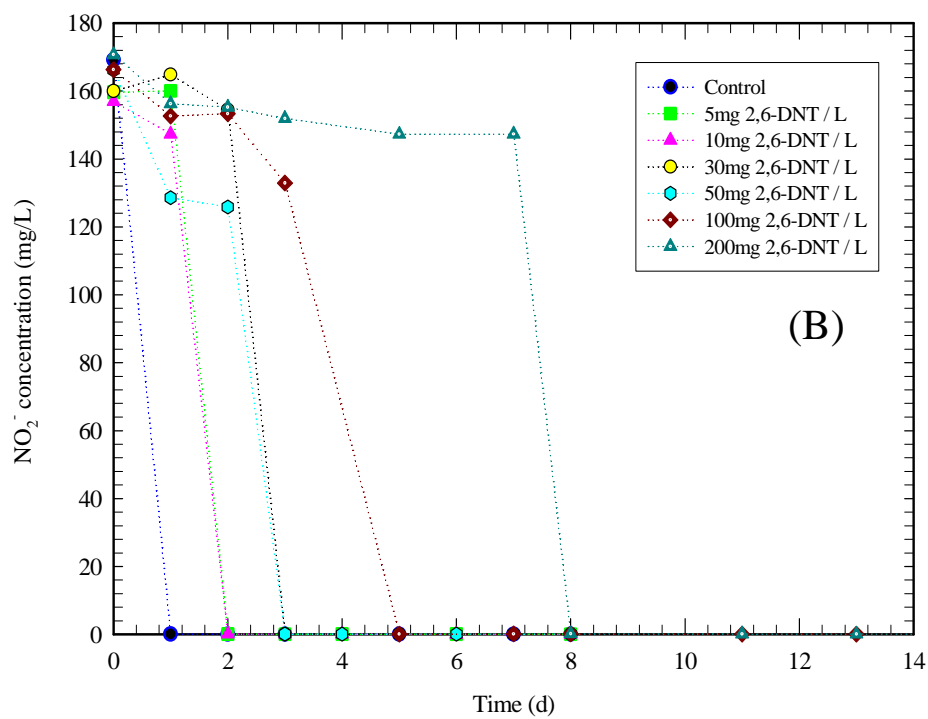
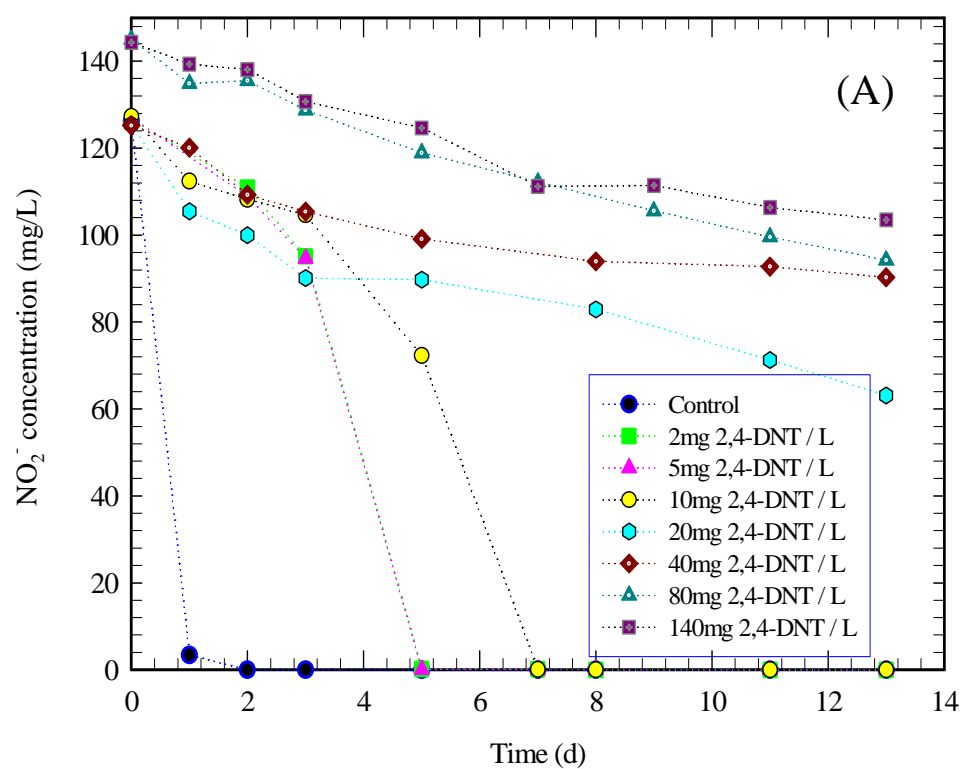
DNT inhibition on denitrification was evaluated in shake flasks spiked with various DNT concentrations. Results indicate that denitrification rate and extent are effected by increasing 2,4-DNT and 2,6-DNT concentration. Figure 5.4A presents that even a minimal level of 2,4-DNT resulted in slower denitrification rates, becoming severely limiting at 20 mg/L and above. Nitrite in negative control reactors was reduced completely after 3 days. Parallel experiments for 2,6-DNT resulted in a longer lag phase before denitrification at concentrations of 2,6-DNT nearing saturation (Figure 5.4B). However, nitrite levels dropped to near detection limits in the 2,6-DNT systems in



**Figure 5.2.** Nitrogen profiles observed in denitrification shake flasks with an acclimated denitrifying culture (after 1 month enrichment).



**Figure 5.3.** Typical ratio of COD required for nitrogen reduction during the denitrification studies.



**Figure 5.4.** The effect of (A) 2,4-DNT and (B) 2,6-DNT on denitrification (Inoculation with acclimated denitrifying culture).

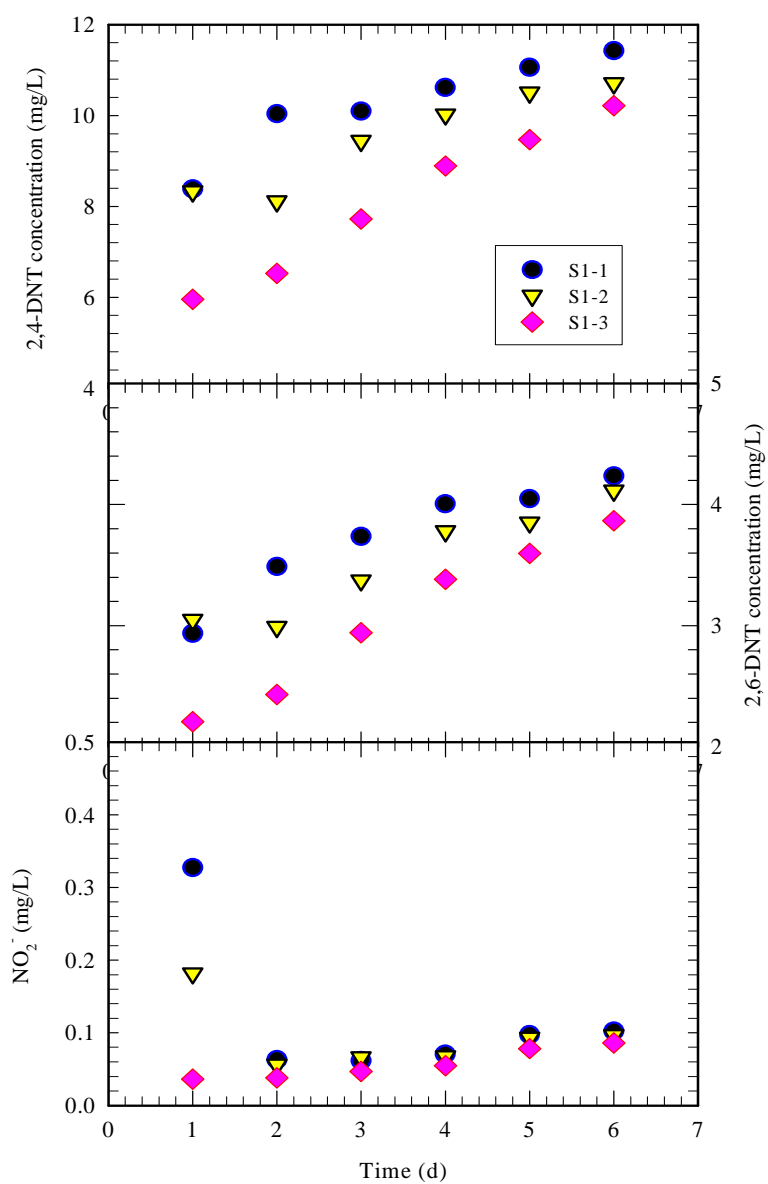
contrast to similar 2,4-DNT amended reactors where denitrification was not observed. At similar concentrations, the rate of denitrification was faster in the presence of 2,6-DNT than 2,4-DNT. Taken together, results indicate that both DNT isomers inhibit denitrification, with 2,4-DNT exerting a stronger inhibitory effect on this denitrifying culture.

#### **5.4.2. Baseline Pilot System Studies.**

Baseline Pilot System studies were conducted to determine constituent concentrations (*i.e.*, 2,4-DNT, 2,6-DNT, and  $\text{NO}_2^-$ ) anticipated during system startup. Data demonstrating baseline concentrations for System 1 are presented in Figure 5.5. Low concentrations of 2,4-DNT and 2,6-DNT were observed and trace amount of nitrite was identified at all sampling points. The same monitoring event was carried out for System 2 (Figure 5.6), observing 2,4-DNT, 2,6-DNT, and  $\text{NO}_2^-$  at similar baseline values after 6 days. It is of note that below average temperatures (0–8 °C) were experienced during this study. A baseline concentration of COD was observed at approximately 130 mg/L for both systems. This was attributed to soluble organics released from the soils in addition to the low baseline concentration of DNT isomers. Appreciable nitrate production was not observed for either during baseline studies.

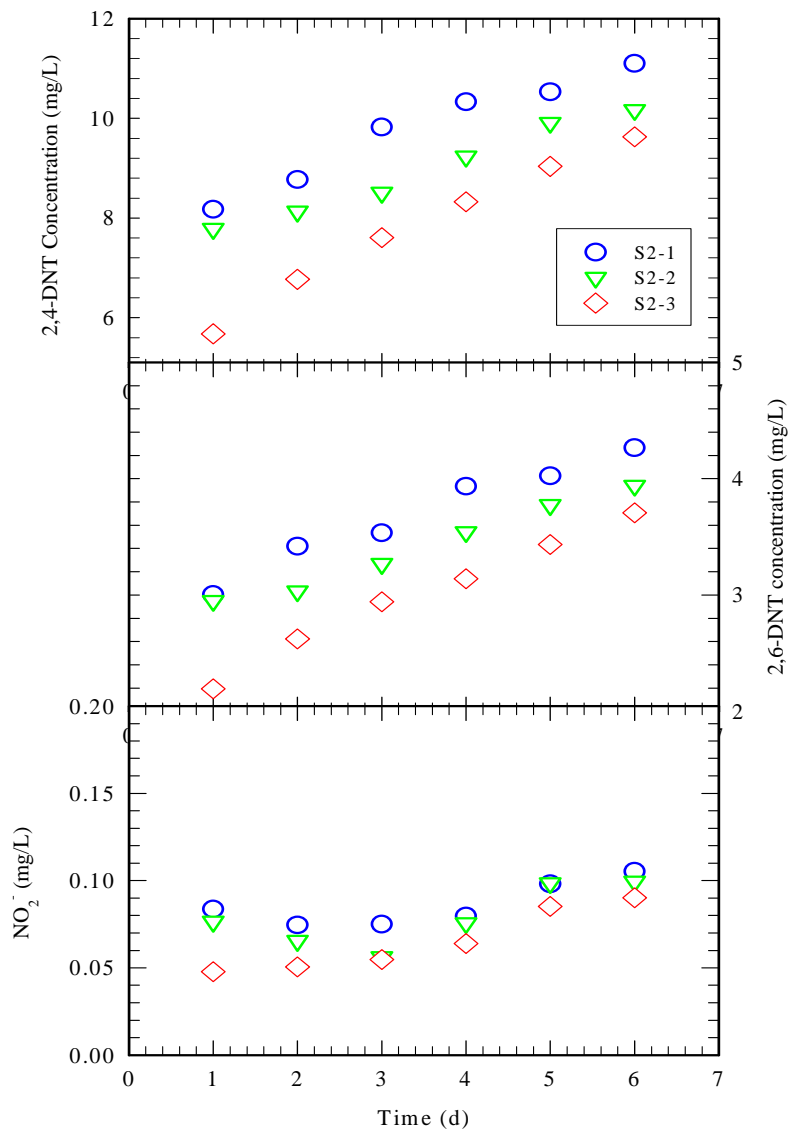
Two conclusions were drawn from these baseline studies. First, relatively low concentrations of DNT isomers were leaching from the soil and DNT concentrations at each sampling point stabilized at similar values suggesting that DNTs were not retained on materials in the pilot system. Second, biodegradation of DNT was minimal during baseline study as little production of nitrite/nitrate was observed. Based on these

conclusions, the levels of each DNT isomer were used as guidance for reactor operation upon the onset of biodegradation.



**Figure 5.5.** Baseline concentrations of 2,4-DNT, 2,6-DNT, and nitrite in Pilot System 1.





**Figure 5.6.** Baseline concentrations of 2,4-DNT, 2,6-DNT, and nitrite in Pilot System 2.

#### **5.4.3. Screening of Denitrification Activity in DNR Tanks.**

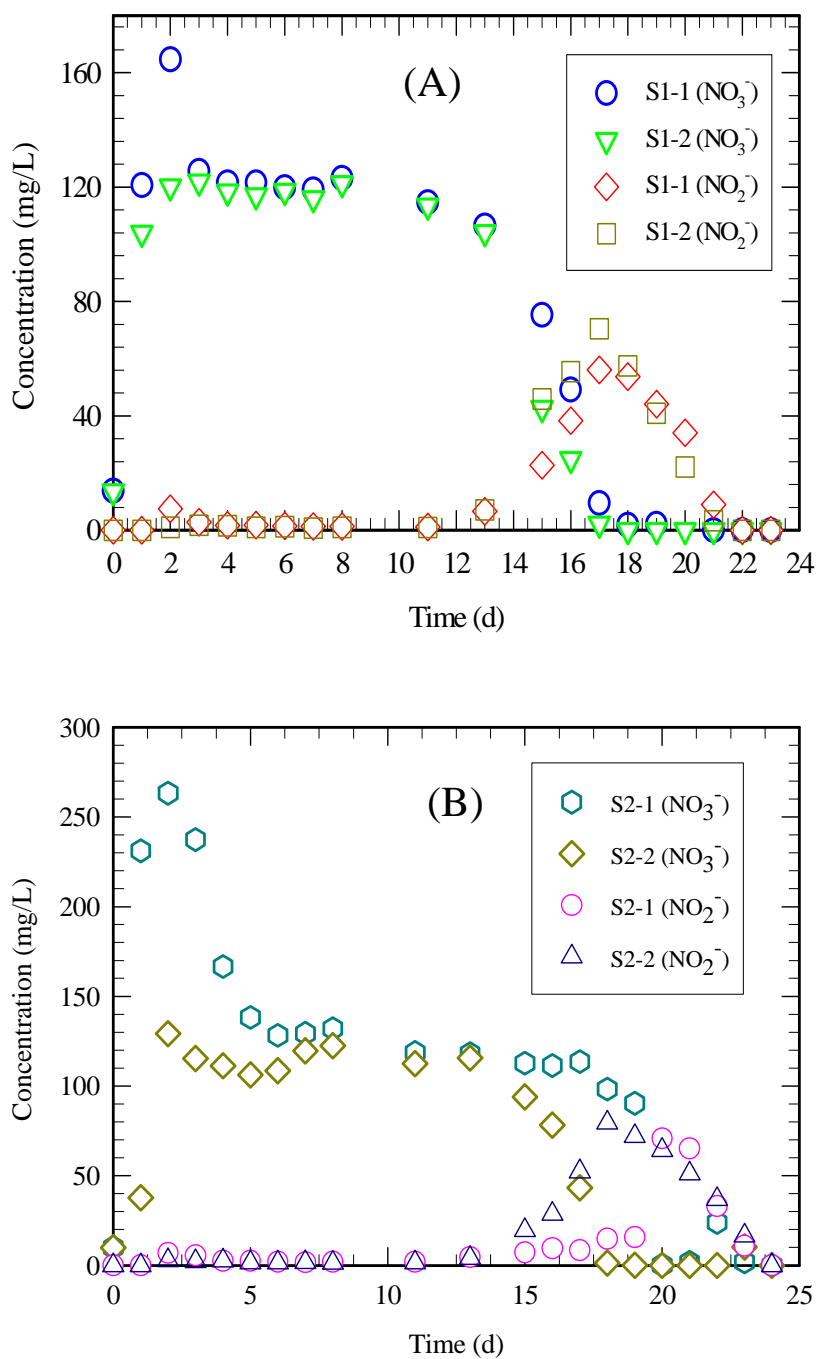
DNR tanks were inoculated with denitrifying cultures enriched in batch systems. Figure 5.7 presents profiles of nitrite and nitrate concentrations in DNR tanks of System 1 and 2 during startup. There was no addition of external carbon source before 10 d, whereby dextrose was added and nitrogen levels then decreased. Commensurate with the onset of nitrate reduction, the level of nitrite was observed to increase and then decrease again. This suggest that acclimation of denitrifying cultures was active (10). The pH in DNR tanks increased from 6.6 to 7.2, from alkalinity produced during heterotrophic denitrification (1, 8, 10). Observations were consistent for both systems.

#### **5.4.4. Integrated BioPilot Studies (DNT Mineralization-Denitrification)**

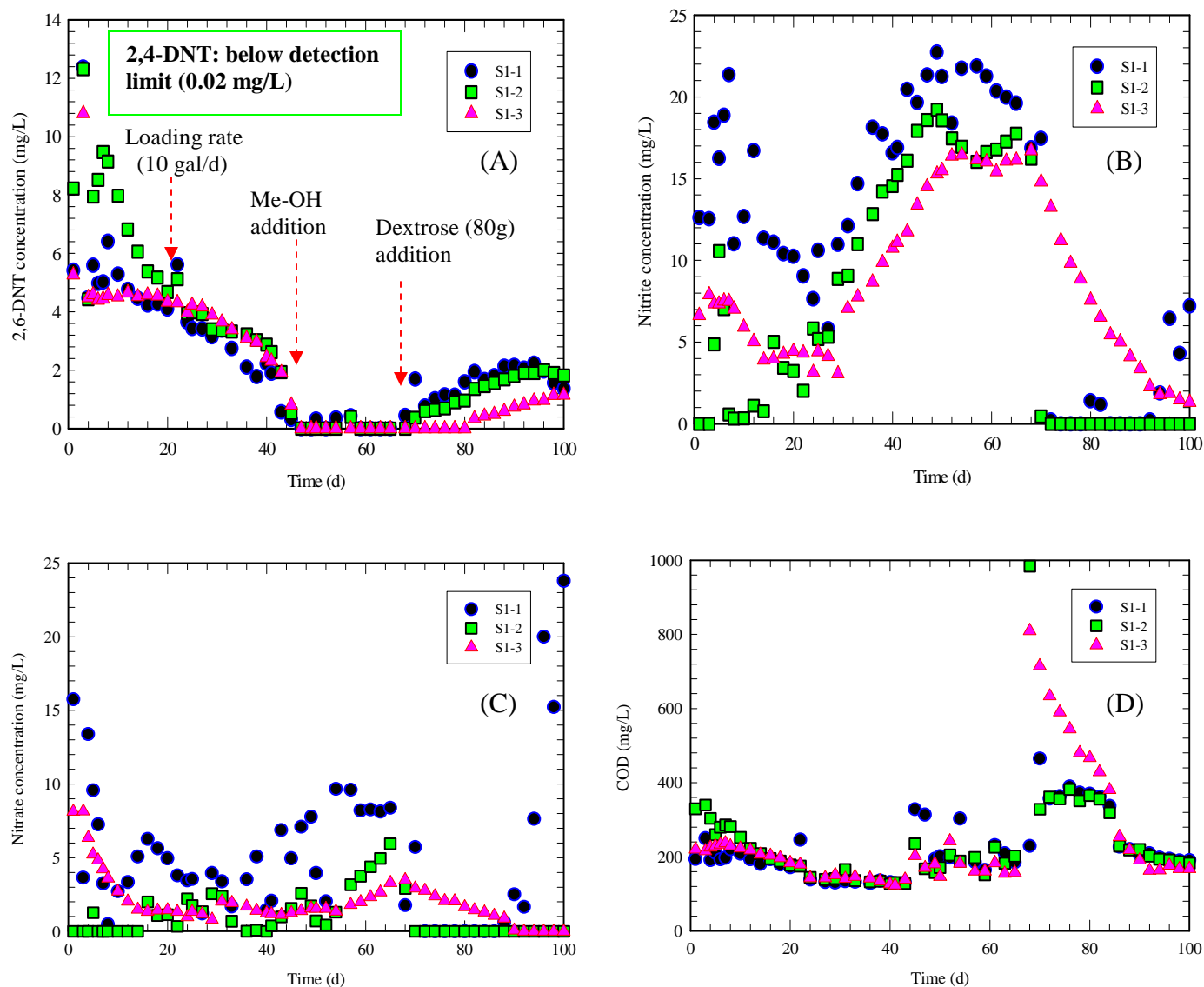
##### **5.4.4.1. DNT, Nitrogen, and COD Profiles in System 1.**

Figure 5.8 presents the effluent concentrations of 2,6-DNT,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , and COD over time in Pilot System 1. Effluent 2,4-DNT was below detection limits (0.02 mg 2,4-DNT/L) at all sampling points over the entire 100 day operation. 2,6-DNT was immediately identified at all sampling points, ranging from 4.10 to 8.23 mg 2,6-DNT/L at outlet of soil tank over the first 23 d of operation (Figure 5.8A). On day 24, the influent loading rate to system 1 was changed from 20 to 10 Gal/d to increase the contact time. The level of 2,6-DNT in each sampling point dropped slowly and approached detection limits after 45 d of operation. The pH of effluents was appropriate for DNT degradation (typically 6.71 to 8.05) throughout the study.

Nitrite and nitrate production from soil tanks was used as an indicator of DNT biodegradation (3, 5, 23, 24). Nitrite was released from soil tank and fluctuated from 5.8



**Figure 5.7.** Nitrite and nitrate profiles observed at DNR tanks of System 1 (A) and System 2 (B) during enrichment of denitrification cultures.

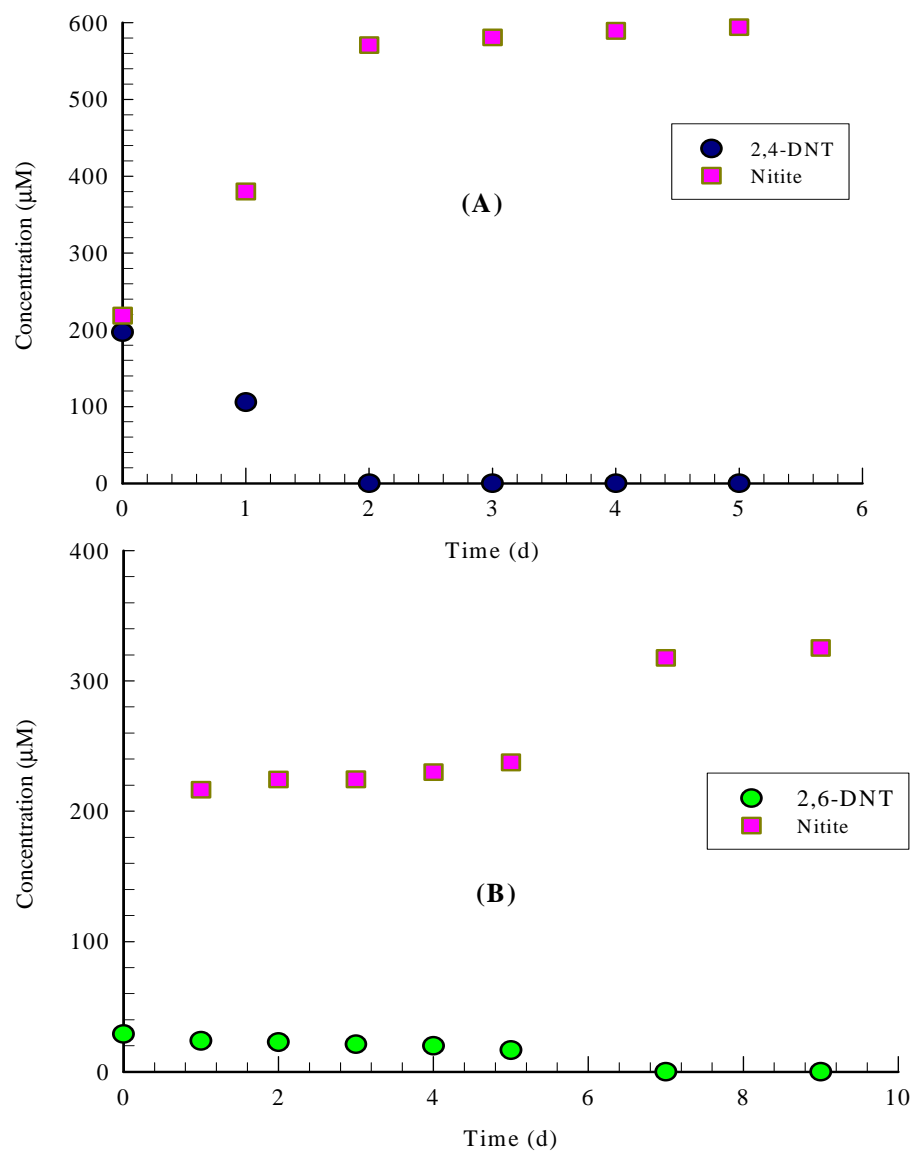


**Figure 5.8.** The profiles of effluents in system 1: (A) 2,6-DNT effluent concentrations. (B)  $\text{NO}_2^-$  effluent concentrations (C)  $\text{NO}_3^-$  effluent concentrations (D) COD effluent concentrations. 1, 2, and 3 represent sequential tank sampling ports for each system.

to 21.4 mg NO<sub>2</sub><sup>-</sup>/L over 23 d of operation (Figure 5.8B). After the reduction of the loading rate, nitrite levels in the outlet of Soil Tank 1 increased gradually with corresponding a degradation of 2,6-DNT. Similar performance was observed in the effluent nitrate concentrations over the first 23 d operation. After 23 days of operation (before addition of external carbon sources), nitrate levels were stabilized in contrast to rising nitrite concentrations.

In summary, immediate depletion of 2,4-DNT was observed in System 1, with undetectable effluent concentrations remaining throughout the study. Commensurate with a reduction of water loading rate, biodegradation of 2,6-DNT occurred as effluent 2,6-DNT concentrations decreased with an increase of nitrite evolution. Effluent shake flask studies were conducted to confirm the presence and activity of 2,4-DNT and 2,6-DNT degraders from the soil tank (5). Results of these studies (Figure 5.9) show that shake flask spiked with 36.4 mg 2,4-DNT/L was depleted within 2 d below the detection limit and evolved stoichiometric nitrite concentrations. The loss of 2,6-DNT (after 7 d) was also observed in shake flasks. These studies were carried out at 22 °C. Identical shake flask studies were also conducted at a lower temperature (4 °C) and same results, albeit slower, were observed for both DNT isomers (data not shown).

Nitrite and nitrate depletion from DNR tank served as the primary indicator of denitrification (10, 25). Initial nitrite and nitrate levels were near detection limits at outlet of DNR tank, but nitrite concentration increased steadily until day 50. Nitrite levels increased (ranged from 3.1 to 14 mg NO<sub>2</sub><sup>-</sup>/L) with a small increase in nitrate (1.4

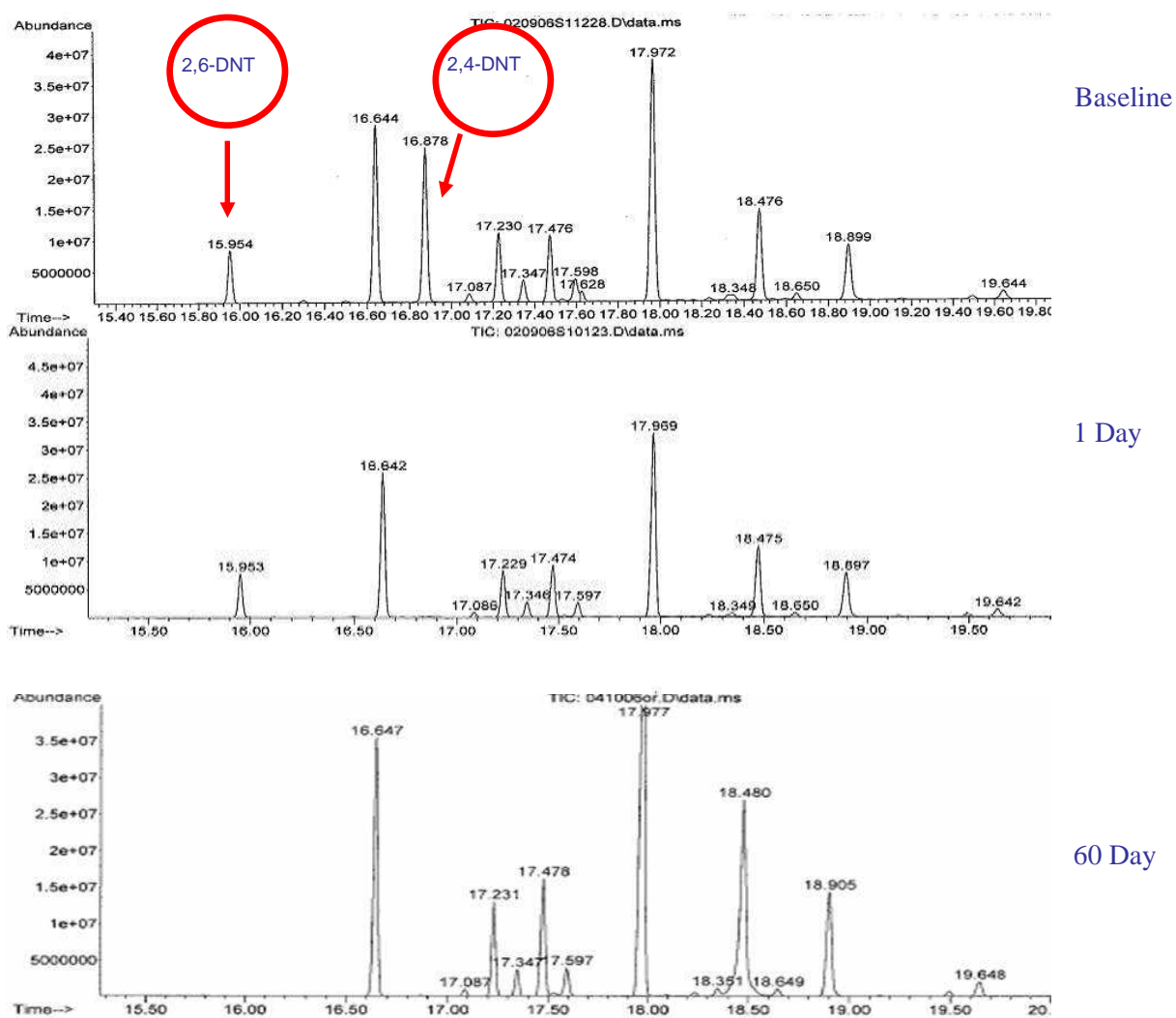


**Figure 5.9.** Pilot effluents shake flask studies at 22 °C (taken from day 1 effluent, system 1). **(A)** Results from 2,4-DNT amended reactor **(B)** Results from unamended reactor.

mg NO<sub>3</sub><sup>-</sup>/L in the second stage (from day 23 to day 45)). The elevation of nitrogen levels in DNR tank indicated the lack of activity of the denitrification system.

As observed for the DNR tank upon the reduced of loading rate, we sought to evaluate which factor(s) caused the absence of the denitrification processes. It was postulated that all biodegradable COD originally present had been consumed at this juncture in the study and that the remaining COD was recalcitrant. Profiles of COD(carbon and energy sources) concentrations were monitored as presented in Figure 5.8D. More COD than baseline levels (130 mg/L) were present in initial integrated system due to additional external carbon source amended during denitrifying enrichment in the DNR tank. COD concentrations decreased steadily, and then stabilized to levels observed in the initial baseline. This demonstrates that recalcitrant substrates in the system were responsible for baseline COD and appeared to be non-biodegradable. Recalcitrant compounds (*i.e.*, dinitroxylenes, TNT, *etc.*) were, in part, observed by GC-MS (Figure 5.10). Such material resulted in an unusable COD/N ratio as the COD was not readily degradable.

For appreciable denitrification in DNR tank to occur, the addition of biodegradable carbon and energy source was necessary (8, 10, 25, 26). Methanol and dextrose were employed as an external source based on a ratio of the COD to NO<sub>3</sub>-N (COD/NO<sub>3</sub>-N = 8–10) obtained during preliminary studies. In System 1, methanol was employed first on day 45 (Figure 5.8). The level of COD increased slightly and nitrite concentrations remained stable after the addition, whereby the level of nitrate increased marginally. It is not clear that the activity of denitrifying bacteria was recovered by addition of methanol.



**Figure 5.10.** GC-MS results of baseline and integrated operation (System 1).



A large amount of dextrose (80 g) was employed as carbon source replacing methanol on day 68, which resulted in the immediate depletion of nitrite and nitrate in DNR effluent sample. COD concentrations averaged 365 mg/L and 352 mg/L respectively and biodegradable COD began flowing to the recycle tank. As shown in Figure 5.8A, 2,6-DNT effluent concentrations at all sampling points increased after the dextrose addition. This, in turn, resulted in less nitrite and nitrate being released, and a reduction of COD utilized. As COD levels decreased again after 86 days, considerable nitrite and nitrate were released at soil tank, and 2,6-DNT level also began to decrease. Nitrite and nitrate levels in the DNR tank were no longer detected.

In the recycle tank, steady 2,6-DNT concentrations (average 4.5 mg/L) were observed over the first 23 d operation, and then decreased slowly after the change of infiltration rate. Initial nitrite concentrations were 6.6 mg/L, and stabilized at 4.2 mg/L. Nitrate levels decreased (from 8.2 to 1.0 mg NO<sub>2</sub><sup>-</sup>/L). After addition of external carbon source, the profiles of DNT isomers, nitrite, nitrate, and COD in recycle tank were similar to those from other sampling points. Depletion of nitrite was not observed in spite of long hydraulic retention time (approximately 10 d), thus ruling out potential nitrite oxidation occurring. This is hypothesized to be the result of inhibition of nitrifiers by recalcitrant COD, which is comprised of various nitroaromatic materials (*i.e.*, dinitroxylenes, *etc.*)

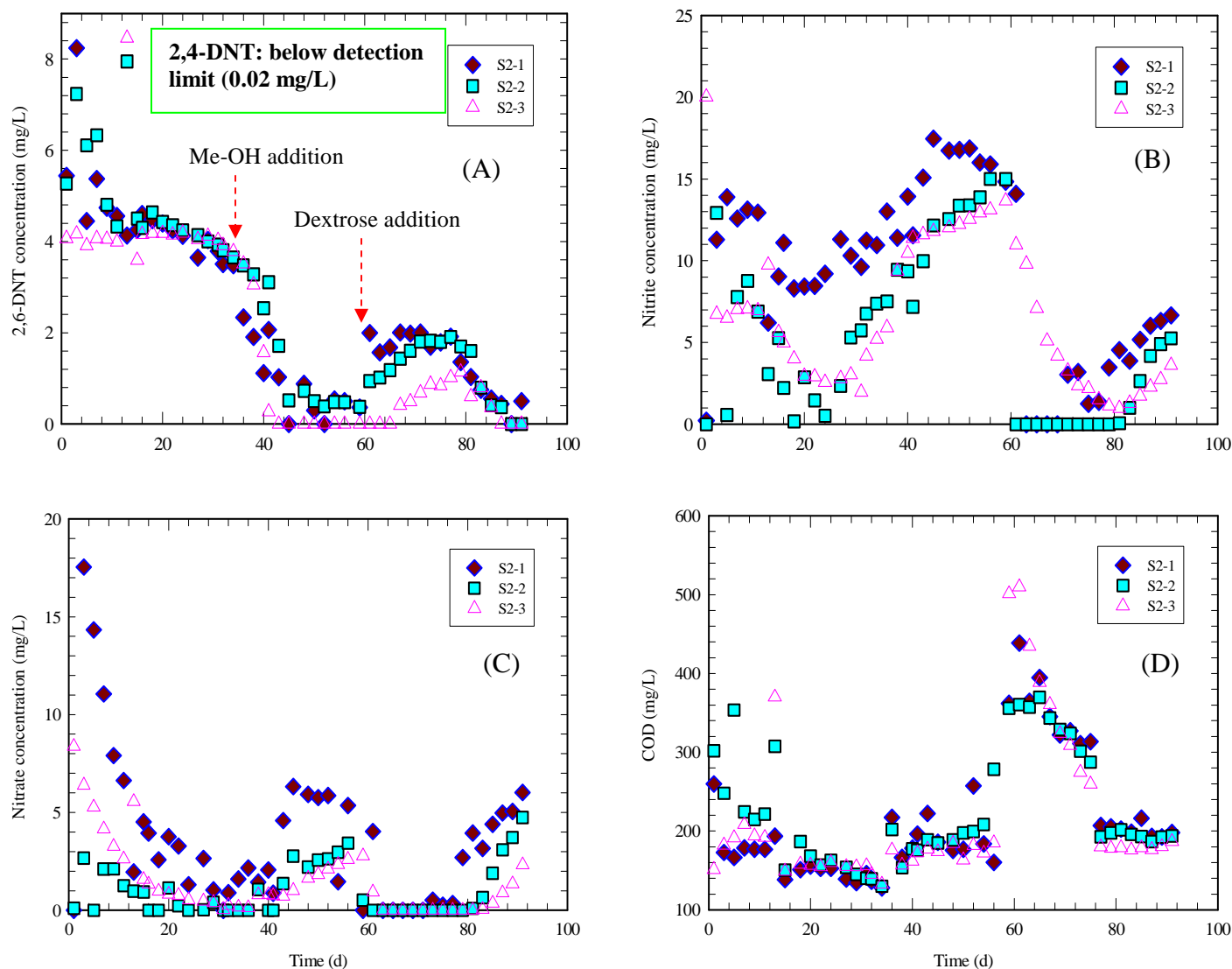
#### **5.4.4.2. DNT, Nitrogen, and COD Profiles in System 2.**

The experimental protocol in System 2 was identical to that of System 1, except that the intermittent flow rate to the soil tank was maintained constantly at 20 Gal/d.

Results (Figure 5.11) demonstrate that DNT,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , and COD paralleled those observed in System 1. Over the first 24 d of operation, 2,6-DNT effluent concentrations from each sampling point stabilized to the similar levels obtained at System 1 (Figure 5.11A) where the level of nitrite and nitrate decreased initially, and increased again (Figure 5.11B and C). The COD levels eventually equilibrated at approximately 153 mg COD/L (Figure 5.11D). On day 35, methanol as external substrate (COD) was employed to the bottom of DNR, which lead to increase of COD level. However, from the DNR tank, levels of nitrite and nitrate increased over 25 days of operation. Dextrose (80 g) was added on day 59 as conducted for System 1, which resulted in increase of 2,6-DNT and depletion of nitrite and nitrate. As degradable COD was depleted to baseline COD observed, 2,6-DNT decreased again, and nitrite and nitrate increased. For all sampling events, 2,4-DNT was never detected for System 2 over 100 days of operation.

Several conclusions can be derived from the comparison of the two pilot studies. Rapid biodegradation of 2,4-DNT was initiated and maintained by simply providing appropriate moisture. Previous studies have shown that 2,4-DNT biodegradation can be limited based on oxygen, nutrient, bi-products, and/or buffer limitations. There were no observed critical factors limiting 2,4-DNT biodegradation in the pilot systems upon the onset of infiltration.

2,6-DNT biodegradation was not initiated for several weeks and degradation rates were slower than 2,4-DNT, but enhanced nitrite production was identified as indicator of 2,6-DNT biodegradation. DNT degradation was initiated and sustained without any nutrient addition or pH adjustment in comparison to Fortner's studies (5) where nutrients



**Figure 5.11.** The profiles of effluents in System 2: (A) 2,6-DNT effluent concentrations. (B)  $\text{NO}_2^-$  effluent concentrations (C)  $\text{NO}_3^-$  effluent concentrations (D) COD effluent concentrations. 1, 2, and 3 represent sequential tank sampling ports for each system.

were required. Additionally inhibition of 2,6-DNT degradation by way of 2,4-DNT (in soils) was not observed with several weeks of startup.

The low infiltration rate of System 1 did not increase the bioactivity of 2,6-DNT degradation in comparison to System 2. In previous column studies (Chapter 4), 2,6-DNT biodegradation was influenced by hydraulic retention time. Presumably this is the result of different experimental conditions (*e.g.*, water recirculation, the presence of recalcitrant nitroaromatics, soil properties and matrix, *etc.*), and understanding the role of retention time requires additional study.

Appreciable COD was present in soil that was not readily biodegradable. The recalcitrant COD could not be used as carbon and energy source for denitrification in DNR tanks. External organic substrates were added to initiate denitrification. The immediate drop of nitrite and nitrate in outlet of DNR tank was the result of denitrification, and was continued as long as biodegradable COD remained. Interestingly, the level of effluent 2,6-DNT increased with addition of an external carbon source, and then decreased again with loss of biodegradable COD. Competition among various oxidizers for the same growth rate limiting factor (*e.g.*, O<sub>2</sub>) is common and the outcome of competition in an open system is dependent of the relationship of the specific growth rates and limiting substrate concentrations (8, 27). The presence of biodegradable COD can inhibit DNT biodegradation, and thus appropriate COD is needed for the complete denitrification but below inhibitory levels to maintain DNT biodegradation if recycle is practiced.

#### ***5.4.5. Identification of Recalcitrant Compounds in Soil Tank Effluents.***

Biopilot systems demonstrated complete depletion of 2,4-DNT after onset of integrated studies and a slower rate of 2,6-DNT degradation as verified by GC-MS. Data of effluent samples in baseline and integrated studies were presented in Figure 5.10. Multiple organic constituents eluted with retention times ranging from 15.9 to 19.6 min. 2,6-DNT and 2,4-DNT eluted at 15.9 and 16.8 min, respectively. Products observed at 16.6 and 17.9 min were identified as dinitroxylenes via MS. These were confirmed through comparison of HPLC-UV spectra in an aqueous phase samples and retention time known standards (data not shown). 1,5-dimethyl-2,4-dinitrobenzene was the predominant dinitroxylenes isomer. These nitroaromatics were repeatedly observed during baseline studies and accumulated over system operation time. Peak disappearance of DNT isomers was observed upon integrated operation, but other nitroaromatics were consistently observed over the sampling events, which account, at least partially, for the baseline recalcitrant COD in aqueous samples.

#### ***5.4.6. Endpoint Assessments: Soil Core and Final Discharge Analyses.***

Soil cores were analyzed upon completion of the system to estimate the remaining DNT isomers for mass balance calculations (Table 5.2). At the onset of the pilot studies, soils contained  $964 \pm 624$  mg/kg of 2,4-DNT and  $50 \pm 35$  mg/kg of 2,6-DNT, referred to as background concentrations. At the completion of the studies, soil samples were taken at six points per soil reactor and extracted. Appropriate 2,4-DNT and trace amount of 2,6-DNT were adsorbed to soil in System 1. In System 2, lower concentrations of DNT isomers were observed. Estimates of DNT mass removal over the experimental period

was conducted based on water volume retained in soil tank and remaining mass of DNT isomers in aqueous phase. Through comparison with background DNT concentration, the efficiency of 2,4-DNT and 2,6-DNT removed were estimated to be 92–95 % and 96–97 %, respectively.

Water in recycle tanks was considered a final discharge point for this operating system. Example data for each constituent of interests is shown in Table 5.3.

Appreciable nitrification processes in both recycle reactors were not observed over 100 days of operation. Recycle tanks were continued to run as batch reactors, after cessation of water flow (at 100 day). After 4 months (on day 220), minimal nitrite remained and nitrate was produced due to nitrification.

**Table 5.2.** Soil extraction results of 2,4-DNT and 2,6-DNT in the pilot systems.

System	Background DNT (mg/kg) at day 0		DNT concentrations in soil treated during 100 d	
	2,4-DNT	2,6-DNT	2,4-DNT	2,6-DNT
S 1	964 ± 624	50 ± 35	71±9.8	trace
S 2			45±9.0	trace

**Table 5.3.** Effluent constituent concentrations in recycle tanks; Baseline concentration vs. remaining concentration after 100 d treatment (unit: mg/L).

Recycle Tank 1	2,4-DNT	2,6-DNT	Nitrite	Nitrate	COD
Baseline	10.21	3.87	0.09	ND	~ 130
After 100 d (Integrated operation)	ND	1.14	1.32	ND	168
After 220 d (120 days batch)	ND	ND	0.23	17.8	128
Recycle Tank 2	2,4-DNT	2,6-DNT	Nitrite	Nitrate	COD
Baseline	9.63	3.71	0.09	ND	~ 130
After 100 d (Integrated Operation)	ND	ND	3.61	2.33	186
After 220 d (120 days batch)	ND	ND	0.11	25.01	134.5

## 5.5. Conclusions

The results presented that 2,4-DNT and 2,6-DNT were readily biodegraded in soil pilot systems under conditions that simulate water infiltration with 2,4-DNT being the most readily degraded of the two isomers. This suggests that DNTs (2,4-DNT and 2,6-DNT) can be biodegraded to low levels at rates that preclude percolation into the water table and thus *in situ* bioremediation appears a viable remediation strategy for managing DNTs contamination in these soils. Nitrite evolved from DNT degradation was treated due to concerns of nitrite in groundwater. Treatment of nitrogen evolution associated with DNT biodegradation was achieved through the augmentation of degradable COD. This suggests that anaerobic reduction of nitrite and nitrate, in vadose zones, will occur if biodegradable COD is present. However, a loss of DNT degradation activity was recycled into the soil. These findings suggest that suitable control of COD is needed for feasible *in situ* bioremediation containing both DNT biodegradation and denitrification processes if recirculation of water is used.



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## CHAPTER 6

### Natural Attenuation of Dinitrotoluenes: Determination of Biodegradation Limits

#### 6.1. Overview

If natural attenuation (NA) is to be an effective remediation strategy for dinitrotoluene (DNT) contaminant plumes, biodegradation processes must contribute to the destruction of DNT's at low concentrations. The overall objective of studies presented herein was to experimentally determine the lowest concentrations of DNT that would support sustained growth of DNT degrading mixed cultures and assess the potential for biodegradation to facilitate successful NA approaches. Previous reports suggest that DNT biodegradation may not support sustained growth at concentration needed for acceptable risk reduction (*i.e.*, regulatory limits).

The direct measurement of degradation limits for each DNT isomer was conducted in both suspended and attached growth systems. After reaching steady state biodegradation levels, the influent concentrations to experimental systems were reduced in a stepwise manner. This process was repeated until the concentration in the effluent was neither influenced by the concentration in the influent, nor the hydraulic retention time. The minimum achievable 2,4-DNT concentrations for chemostat and column system were  $0.052 \pm 0.002 \mu\text{M}$  and  $0.060 \pm 0.004 \mu\text{M}$ , respectively. For 2,6-DNT, the minimum value for chemostat studies was  $0.035 \pm 0.004 \mu\text{M}$ , and in columns the minimum value achievable was  $0.026 \pm 0.004 \mu\text{M}$ . These experimentally determined threshold values for DNT isomers are much lower than those predicted from studies conducted with high levels of DNT contamination and suggest that NA that includes

active biodegradation is capable of reaching risk reduction in levels needed in groundwater.

## 6.2. Introduction

Remediation of DNT contaminated media is costly and time consuming, thus passive natural attenuation (NA) strategies employing biological destruction of DNT present an attractive remediation alternative. While soil concentrations often vary by site history, long-term DNT persistence is often observed and these contaminated soils have the potential to be long term source of groundwater contamination. Little information exists regarding DNT NA in contaminant plumes or the effectiveness of biological degradation at low DNT concentrations.

One method of assessing biodegradation limits is through the calculation of minimum substrate concentration ( $S_{\min}$ , equation 6.1)) using DNT biodegradation kinetic parameters obtained from previous studies (1-3).

$$S_{\min} = \frac{K_s \times b}{Yk - b} \quad (6.1)$$

In equation 6.1,  $Y$  ( $\mu\text{mol biomass}/\mu\text{mol DNT}$ ) is the true yield coefficient;  $b$  ( $\text{time}^{-1}$ ) is the specific decay rate;  $k$  ( $\mu\text{mol DNT}/\mu\text{mol biomass} \cdot \text{time}$ ) is the maximum specific substrate utilization rate; and  $K_s$  ( $\mu\text{M}$ ) is the Monod half-velocity coefficient. These kinetics can be calculated by fitting experimental data to Monod functions used in the microorganism growth rate expressions (4). Predicted and experimentally derived biokinetic parameters for DNT degradation are provided in Table 6.1. Heinze *et al.* (2)

**Table 6.1.** Estimation of biokinetic parameters based on literature data.

	$S_{\min}$ ( $\mu\text{M}$ )	$Y$ ( $\mu\text{molX}/\mu\text{molDNT}$ )	$k$ ( $\mu\text{molDNT}/\mu\text{molX}\cdot\text{d}$ )	$K_s$ ( $\mu\text{M}$ )
Heinze <i>et. al.</i>	0.44–1.33 (2,4-DNT)	0.48	4.92	10–30
Smets <i>et. al</i>	0.1–1.25 (2,4-DNT)	0.50	0.51–0.61	0.16–1.98
	0.56–2.26 (2,6-DNT)		0.09–0.21	1.15–4.61
Daprato <i>et. al.</i>	0.21–0.91 (2,4-DNT)	0.48	2.5–10	10

used batch cultures for the determination of kinetic coefficients, and a mixed culture simultaneously degrading 2,4-DNT and 2,6-DNT was employed in kinetic studies done by Smets *et al.* (3). Daprato *et al.* modeled the kinetic parameters in slurry reactors, and obtained a range of kinetic parameters with the variation accounted for by a population shift during extended operation (1). Estimates of  $S_{\min}$  using Equation 6.1 and these parameters result in a  $S_{\min}$  value higher than current regulatory requirements (Table 6.2). However, these calculated  $S_{\min}$  values may not be applicable for NA estimations as previous DNT degradation studies were obtained from regression data obtained in experiments conducted at DNT concentrations much higher than expected in groundwater plumes and in systems that are quite different from porous media.

The objective of studies presented herein is to evaluate directly the lowest concentrations of DNT that support sustained growth of DNT degrading mixed cultures under slow growth scenarios. The direct measurement of biodegradation limits for each DNT isomer was carried out in suspended and attached growth systems (chemostat and column systems) respectively. Results of these studies suggest that biodegradation can be effective at achieving DNT destruction at levels much lower than predicted, and that NA may present a viable approach to plume management if conditions for biodegradation are appropriate.

## **6.3. Materials and Methods**

### **6.3.1. Chemicals.**

2,4-dinitrotoluene (97 %), 2,6-dinitrotoluene (98 %), and other chemicals were commercial grade and were purchased from the Fisher Scientific and Sigma-Aldrich Company. Other chemicals and analytical organic solvents were described in Chapter 3.

**Table 6.2.** Regulatory requirements for drinking water and industrial waste streams.

	<b>2,4-DNT</b>	<b>2,6-DNT</b>
CFR	320 µg/L	560 µg/L
EPA drinking water criteria level	50 µg/L	40 µg/L

CFR: Code of federal regulation (40 CFR 268.48), Industrial waste stream

EPA drinking water criteria number: For increased cancer risk



### **6.3.2. Media preparations.**

All components and compositions used in mineral media were described in Chapter 4.

### **6.3.3. Analytical Methods.**

High Pressure Liquid Chromatography (HPLC) analysis was performed with a Hybercarb® porous graphite column (5). For sample concentrations below conventional detection limits, a solid phase extraction (SPE) method was employed using reverse phase liquid chromatography (RPLC: Supelco C<sub>18</sub> columns) (6). Primary separations used a LC-18 (1 mL) column eluted with methanol, ACN, and ACN-water (1:9, v/v) in order. Samples and standards were introduced with ACN (sample:ACN = 9:1). Finally, 0.5 mL of pure ACN was eluted to take an extracted sample after drainage of 0.5 mL of ACN-water solution. Nitrite concentrations were measured following Standard Methods for Wastewater Treatment method 4500-NO<sub>2</sub><sup>-</sup> (7).

### **6.3.4. Culture Source and Maintenance.**

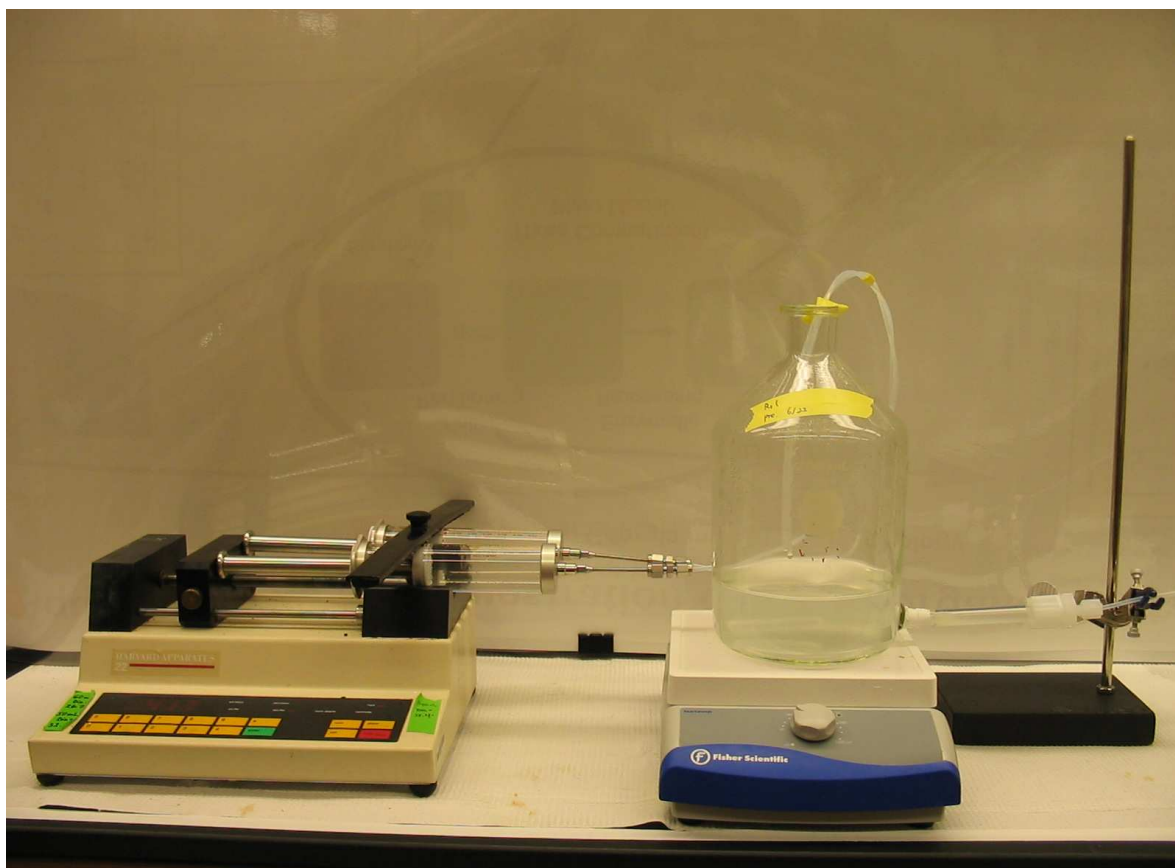
Mixed cultures previously acclimated to individual DNT isomers as the sole carbon and nitrogen source were used in these experiments. Originally cultured from soils (Sources: Badger Army Ammunition Plant, WI for 2,4-DNT and Tyndall Air Force Base, FL for 2,6-DNT), the mixed cultures were maintained in soil free draw and fill reactors using mineral salt medium amended with 2,4-DNT (600 mg/L) or 2,6-DNT (18–90 mg/L). The pH was maintained between 8–8.5, and aliquots from these DNT degrading mixed culture were used to seed experimental systems.

#### **6.3.5. Chemostats.**

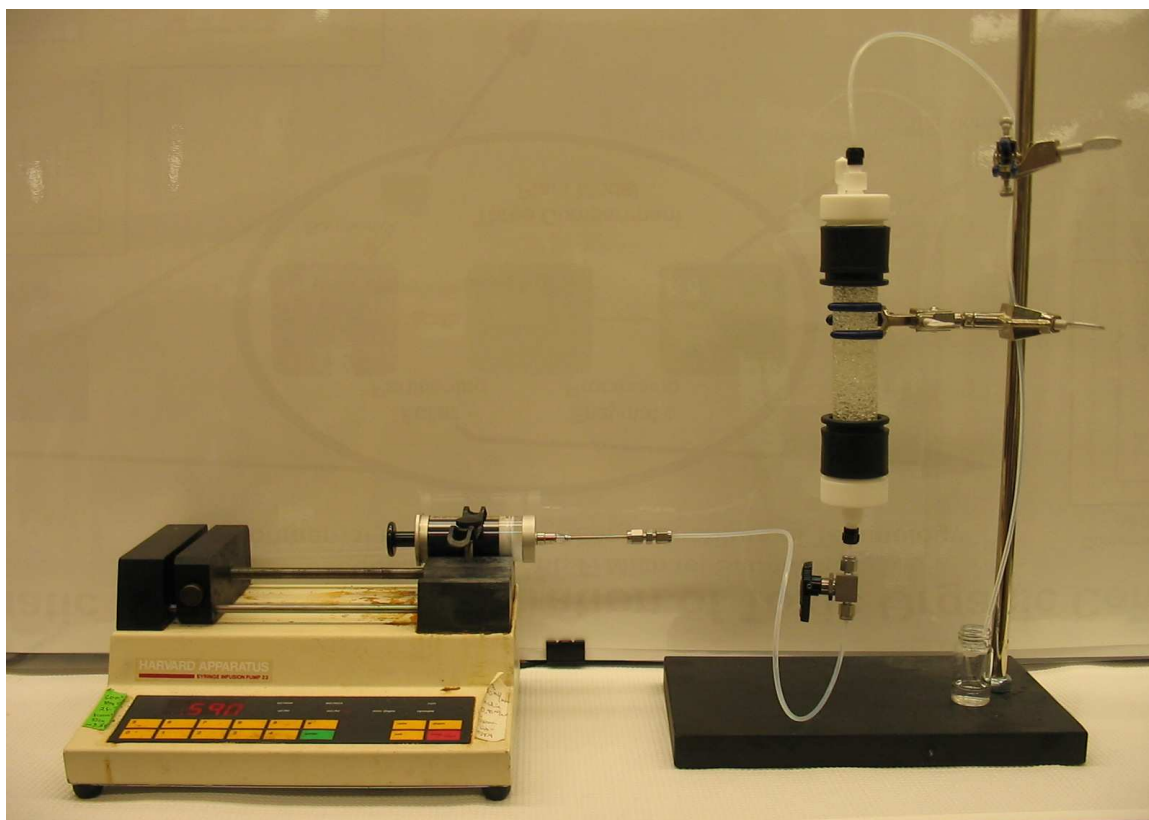
Chemostats were constructed for evaluation of DNT biodegradation limits with flat-bottomed glass vessels (2 L) open to the atmosphere (Figure 6.1). A glass port at the base of the reactor was fitted with Teflon tubing and Teflon reducer to control the flow of reactor effluent (*i.e.*, a fitting was used to reduce the tubing volume from reactor port (1.59 cm) to the effluent (0.32 cm)). With an aqueous volume of 500 mL, HRT was maintained at a 2.5 day. Reactor influent flow of 200 mL/day was provided by a syringe pump, and outflow was hydraulically controlled to maintain a constant volume. Reactors were inoculated by centrifuging 20 mL of the seed culture and washing the cells twice with fresh media to remove DNT and any metabolites. Effluent samples were collected daily and analyzed for nitrite and DNT concentrations.

#### **6.3.6. Column Systems.**

Two columns (*i.e.*, 2,4-DNT column and 2,6-DNT column) were employed, which were 2.5 cm in diameter and 16 cm in length (Figure 6.2). Glass beads were used as the attachment media to minimize complicating factors such as adsorption and desorption of contaminants. Columns were packed dry with a uniform bead distribution. A variable speed syringe pump was used to feed the solution in an up-flow mode. For the evaluation of the column characteristics, the following column parameters were determined: porosity ( $n$ ), pore water velocity ( $v$ ), and dispersion coefficient ( $D$ ). Porosity was determined via a wet method (8, 9). As listed in Table 6.3, the column porosity was similar for both columns. The wet pore volumes were calculated (37.8 and



**Figure 6.1.** Laboratory chemostat schematics for determination of biodegradation limits.



**Figure 6.2.** Laboratory column schematics for determination of biodegradation limits.

35.2 cm<sup>3</sup> for 2,4-DNT and 2,6-DNT columns, respectively) and thus a flow rate of 0.63 and 0.59 mL/hr was employed to maintain a 2.5 d HRT in each. The two important column transport parameters, pore water velocity and dispersion coefficient, were obtained through KBr conservative tracer tests and use of the CXTFIT 2.0 program (8, 9).

Columns were initiated with a feed concentration of an individual DNT isomer at approximately 100 µM, allowing for a biofilm to establish and degradation activity. The feed solution contained 8 mg/L of dissolved oxygen (DO). To adequately sample column effluent, a glass reservoir (10 mL) with a sampling port was connected to the top of the column. Samples withdrawn daily from the effluent reservoir were used to monitor nitrite and DNT concentration.

#### **6.3.7. *Experimental Approach.***

Subsequent to inoculation, a constant feed of DNT was provided in the influent of experimental systems, and effluent concentrations were monitored for nitrite and DNT. Degradation activity was initiated with a feed concentration of individual DNT isomers at 100 µM until steady state was reached (*i.e.*, no variation in reactor concentration or column effluent concentration). After reaching steady state, the influent concentration was reduced to either 20 µM (2,4-DNT) or 50 µM (2,6-DNT). After reduction of influent concentration, the effluent was then monitored to identify the corresponding steady-state concentration. This process was then repeated sequentially at influent concentrations of 5, 1, 0.5, and 0.1 µM, with each reduction to influent concentration occurring after effluent DNT concentrations reached steady state. Biodegradation limits were defined as the

**Table 6.3.** System parameters and operating values for glass bead column used to determine DNT biodegradation limits.

Operation Conditions	
Length of Column	16 cm
Diameter of Column	2.5 cm
Cross-sectional area of Column	4.91 cm <sup>2</sup>
Volume of reactor	78.5 cm <sup>3</sup>
Diameter of glass bead	0.3 cm
Porosity	2,4-DNT Column : 0.48, 2,6-DNT Column : 0.45
Volume of voids	2,4-DNT Column : 37.8 cm <sup>3</sup> , 2,6-DNT Column : 35.2 cm <sup>3</sup>
Feed flow rate	2,4-DNT Column : 0.63 mL/hr, 2,6-DNT Column : 0.59 mL/hr
Retention time	2.5 d

average steady-state reactor concentration where the effluent concentration was not affected by influent concentration or retention time.

## 6.4. Results and discussion

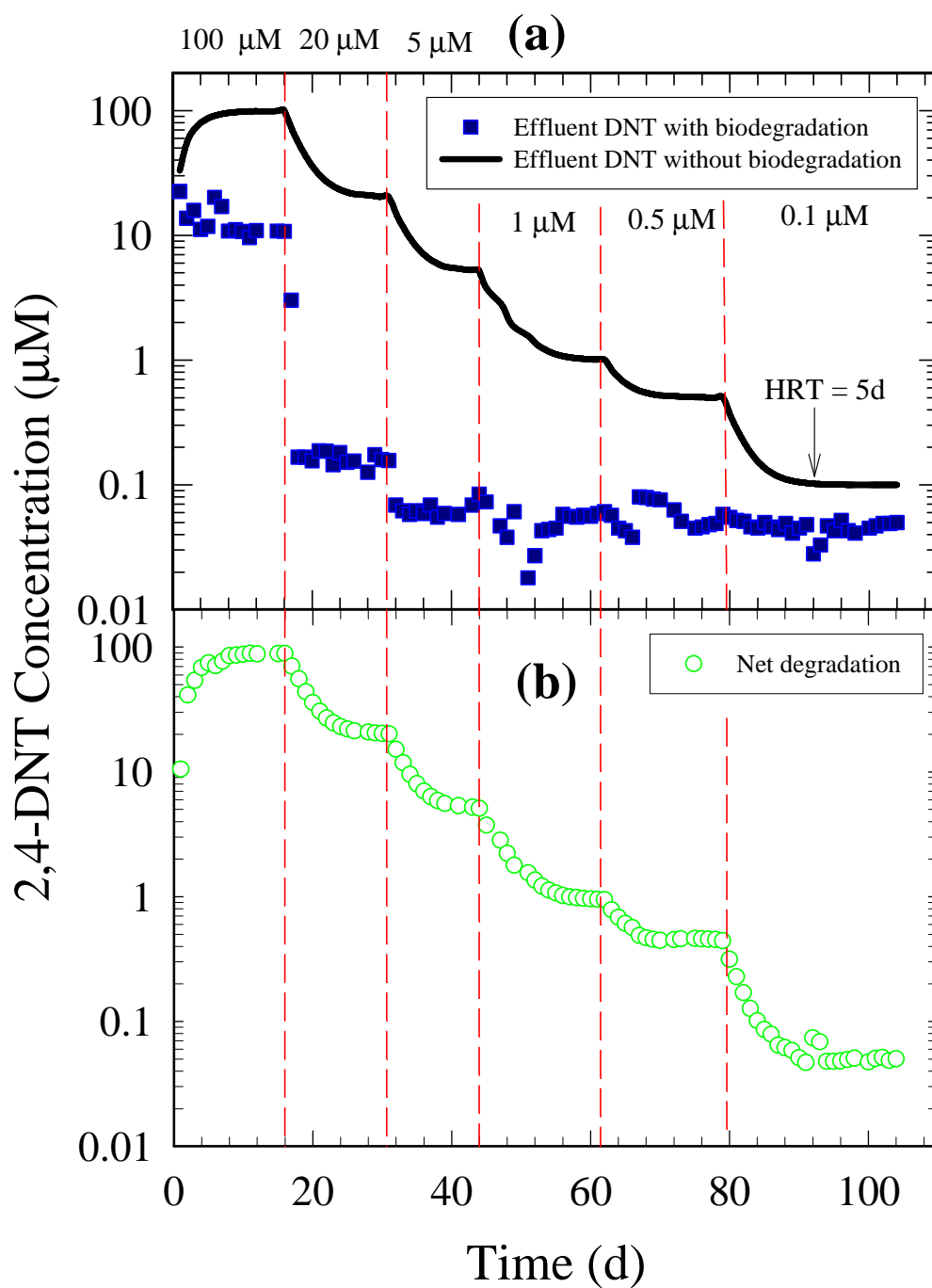
### 6.4.1. Chemostat Studies.

Chemostats were seeded with either a 2,4-DNT or 2,6-DNT degrading mixed culture and monitored until consistent biodegradation was achieved. For 2,4-DNT experiments, the initial influent concentration was 100  $\mu\text{M}$ . After reaching steady state, corresponding to an effluent concentration of  $10.7 \pm 0.48 \mu\text{M}$ , the influent concentrations were reduced in a stepwise manner. Figure 6.3a and 6.3b present the effluent 2,4-DNT concentrations at each influent concentration and the net degradation achieved, respectively. In Figure 6.3a, effluent concentrations are presented in comparison to calculated values that would be expected if no biodegradation was occurring in the chemostat. The concentration profile of a conservative material was predicted considering washout of a chemostat with changing influent concentrations. A mass balance for substrate concentrations (S) through the control volume (V) is:

$$V \frac{dS}{dt} = QS_0 - QS \quad (6.2)$$

After separation of variables and application of boundary conditions becomes

$$\int_{S_{t1}}^{S_{t2}} \frac{dS}{S_0 - S} = \frac{Q}{V} \int_{t1}^{t2} t dt \quad (6.3)$$



**Figure 6.3.** (a) 2,4-DNT effluent concentrations in chemostat for influent concentrations of 100, 20, 5, 1, 0.5 and 0.1  $\mu\text{M}$  DNT. The solid line represents 2,4-DNT concentrations expected without biodegradation. (b) Actual 2,4-DNT degraded in chemostat calculated by difference between experimentally measured and predicted values for 2,4-DNT concentration.



The analytical solution for Equation 6.3 under non-steady state conditions is:

$$S_{t_2} = S_0 - (S_0 - S_{t_1}) \exp\left\{-\frac{1}{\theta}(t_2 - t_1)\right\} \quad (6.4)$$

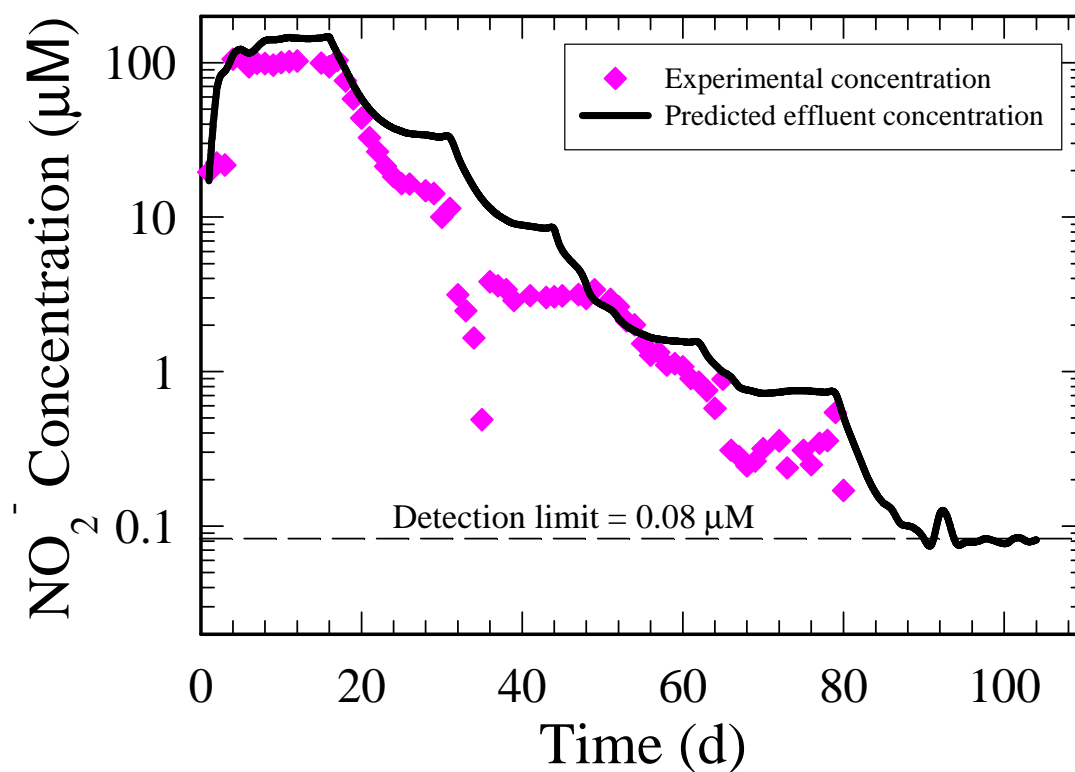
where,  $\theta$  ( $= V/Q$ ) is the hydraulic retention time;  $S_0$  is the feed concentration;  $S_{t_1}$  and  $S_{t_2}$  are resident concentrations in the reactor at time  $t_1$  and  $t_2$  respectively. The difference between this calculated value and the observed value is shown in Figure 6.3b.

Nitrite evolution from 2,4-DNT was monitored to confirm that the loss of 2,4-DNT was due to biodegradation processes. Results of nitrite analyses are presented in Figure 6.4.

Also shown in Figure 6.4 is the concentration of nitrite that would be anticipated from biodegradation using the stoichiometry described by Zhang et al., (1 mole of DNT yields 1.63 moles of nitrite) (10). Stoichiometric quantities of nitrite were observed for 100 and 1  $\mu\text{M}$  2,4-DNT in influent. Deviations from expected values were observed in other influent concentrations as the release of nitrite was less than predicted, and fell below the method detection limit (0.08  $\mu\text{M}$ ) at an influent concentration of 0.1  $\mu\text{M}$  2,4-DNT. Nevertheless, because nitrite production paralleled 2,4-DNT degradation, the system was considered to be actively degrading DNT.

As the influent 2,4-DNT concentration was reduced from 100  $\mu\text{M}$  to 20  $\mu\text{M}$  and then 5  $\mu\text{M}$ , a corresponding decrease in the effluent concentration was observed.

However, the effluent concentrations remained constant at approximately 0.052  $\mu\text{M}$  2,4-DNT when the influent concentrations ranged from 1  $\mu\text{M}$  to 0.1  $\mu\text{M}$ . To further examine if this effluent concentration could be reduced further, the HRT was increased from 2.5 to



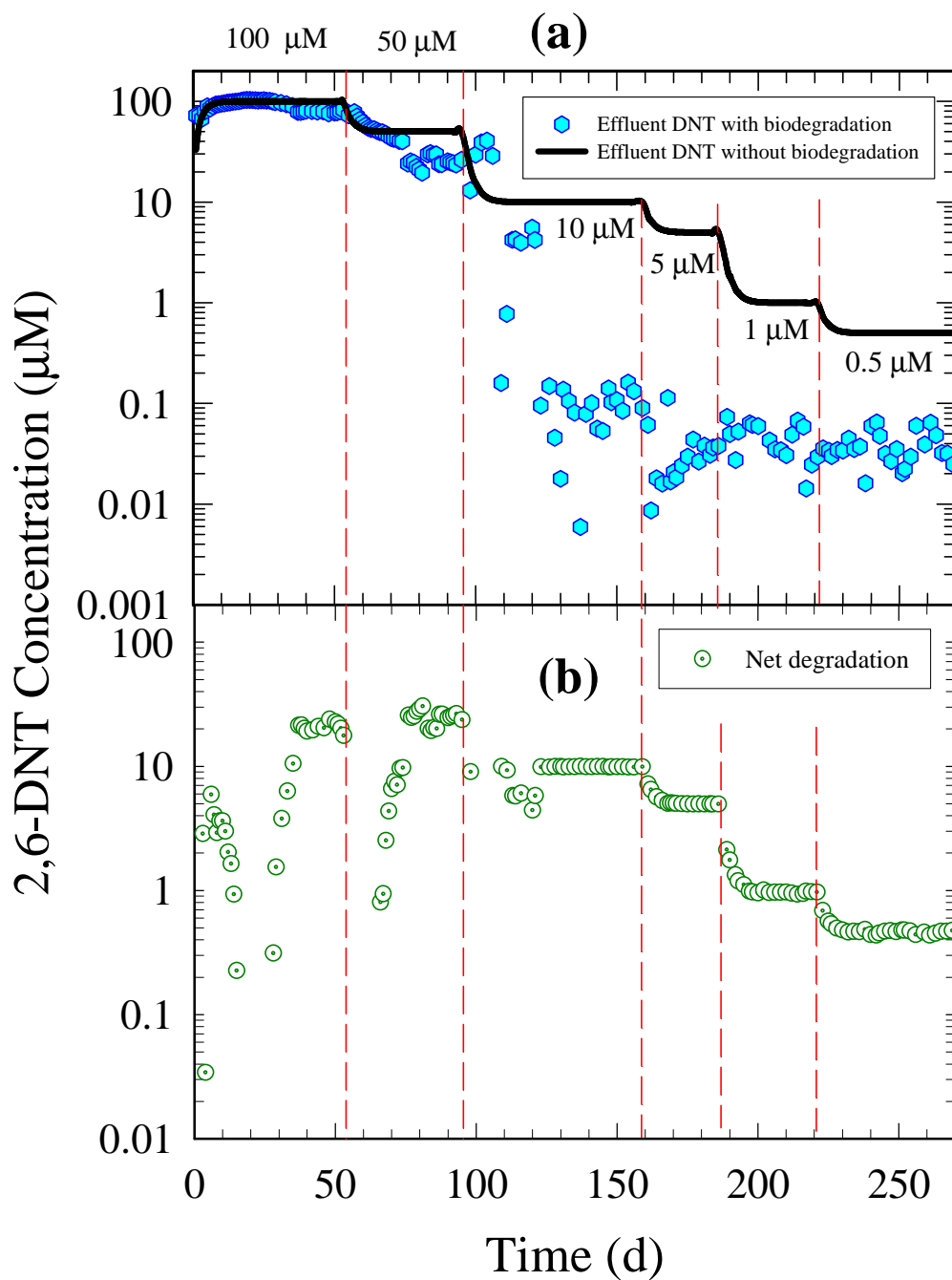
**Figure 6.4.** Experimentally measured values for nitrite concentration in chemostat are presented with time. Expected values for nitrite concentration were calculated based on 2,4-DNT degraded in chemostat and stoichiometry provided by Zhang *et al.*(10).

5 d while maintaining a 0.1  $\mu\text{M}$  influent concentration. No change in effluent concentration was observed with the increased HRT, establishing  $0.052 \pm 0.002 \mu\text{M}$  as the limiting concentration of 2,4-DNT in the chemostat.

The experimental protocol for determining the degradation limits for a 2,6-DNT degrading mixed culture paralleled that outlined for 2,4-DNT. Figure 6.5a and 6.5b show the profile of 2,6-DNT degradation over time at various influent concentrations in the chemostat. 2,6-DNT degraders were known to grow more slowly than 2,4-DNT degraders (11), and the time frame for reaching steady-state in reactor was longer for 2,6-DNT degraders than 2,4-DNT degraders (12, 13). Thus, the experimental time frame was extended to approximately 300 days with typical equilibration time frame ranging from 30 to 60 days.

Increased activity was observed over time including when the lower feed concentrations (50  $\mu\text{M}$ ) were initiated. After steady state was reached, influent concentrations were reduced in a stepwise manner as described previously. The steady-state reactor concentration at 10 and 5  $\mu\text{M}$  influent was reduced to  $0.092 \pm 0.043 \mu\text{M}$  and  $0.031 \pm 0.008 \mu\text{M}$ , respectively. However, the steady-state concentration at low influent concentration (1 and 0.5  $\mu\text{M}$ ) was  $0.039 \pm 0.019 \mu\text{M}$  and  $0.036 \pm 0.014 \mu\text{M}$ . No change was observed in effluent concentration from reactor at longer HRT (from 2.5 to 5 days). As a result, the concentrations in the effluent ( $0.035 \pm 0.004 \mu\text{M}$ ) were no longer influenced by the influent concentration lower than 5  $\mu\text{M}$  of 2,6-DNT, which was established as limiting value in this system.

Experimentally measured nitrite concentrations are shown in Figure 6.6 and compared with expected stoichiometrically derived nitrite values (solid line). There was



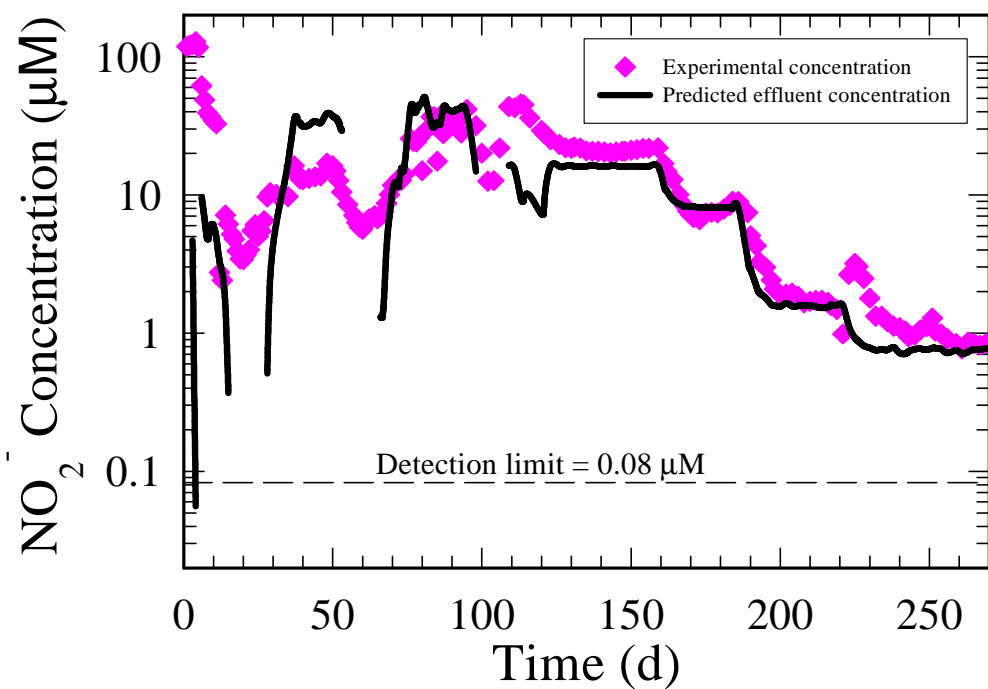
**Figure 6.5.** (a) 2,6-DNT concentrations in chemostat for influent concentrations of 100, 50, 10, 5, 1 and 0.5  $\mu\text{M}$  DNT. The solid line represents 2,6-DNT concentrations expected without biodegradation. (b) Actual 2,6-DNT degraded in chemostat calculated by difference between experimentally measured and predicted values for 2,6-DNT concentration.

no increase in nitrite production with no significant 2,6-DNT decrease over the first period of operation. However, nitrite was increased from the second loading period. For example, when the influent concentration was 50 and 10  $\mu\text{M}$ , the produced nitrite was 33.7 and 21.2  $\mu\text{M}$ , respectively. Subsequently when the influent concentration of 2,6-DNT was reduced to 5, 1, and 0.5  $\mu\text{M}$ , the level of nitrite production was reduced to 7.76, 1.65, and 0.86  $\mu\text{M}$ . These results confirm that nitrite released was associated with 2,6-DNT removal.

#### **6.4.2. Column Studies**

##### **6.4.2.1. 2,4-DNT Column Studies.**

Column studies were initiated by inoculating with mixed cultures able to degrade 2,4-DNT with corresponding nitrite production. Figure 6.7a presents the results of measured effluent concentrations from the column study at six influent concentrations (100, 20, 5, 1, 0.5, and 0.1  $\mu\text{M}$ ). The degradation of 2,4-DNT proceeded rapidly at higher influent concentrations (100, 20 and 5  $\mu\text{M}$  DNT) and steady state effluent concentrations were reached in a relatively short time period (*i.e.*, 3–5 days). For example, when the column was fed a 100  $\mu\text{M}$  2,4-DNT solution, steady state was reached ( $9.50 \pm 0.48 \mu\text{M}$ ) at approximately 4 days. Subsequently, the feed concentration was reduced to 20  $\mu\text{M}$  2,4-DNT, which resulted in the immediate drop of 2,4-DNT and the steady-state concentration to  $0.41 \pm 0.02 \mu\text{M}$ . When the influent concentration was 5  $\mu\text{M}$ , the steady-state concentration in the column effluent was  $0.11 \pm 0.01 \mu\text{M}$ . In this stage, only 3 days were required to obtain steady state concentrations. Further reduction in the influent concentration (1, 0.5, and 0.1  $\mu\text{M}$ ) established a consistent average effluent



**Figure 6.6.** Experimentally measured values for nitrite concentration in chemostat are presented with time. Expected values for nitrite concentration were calculated based on 2,6-DNT degraded in chemostat and stoichiometry provided by Zhang *et al.*(10).

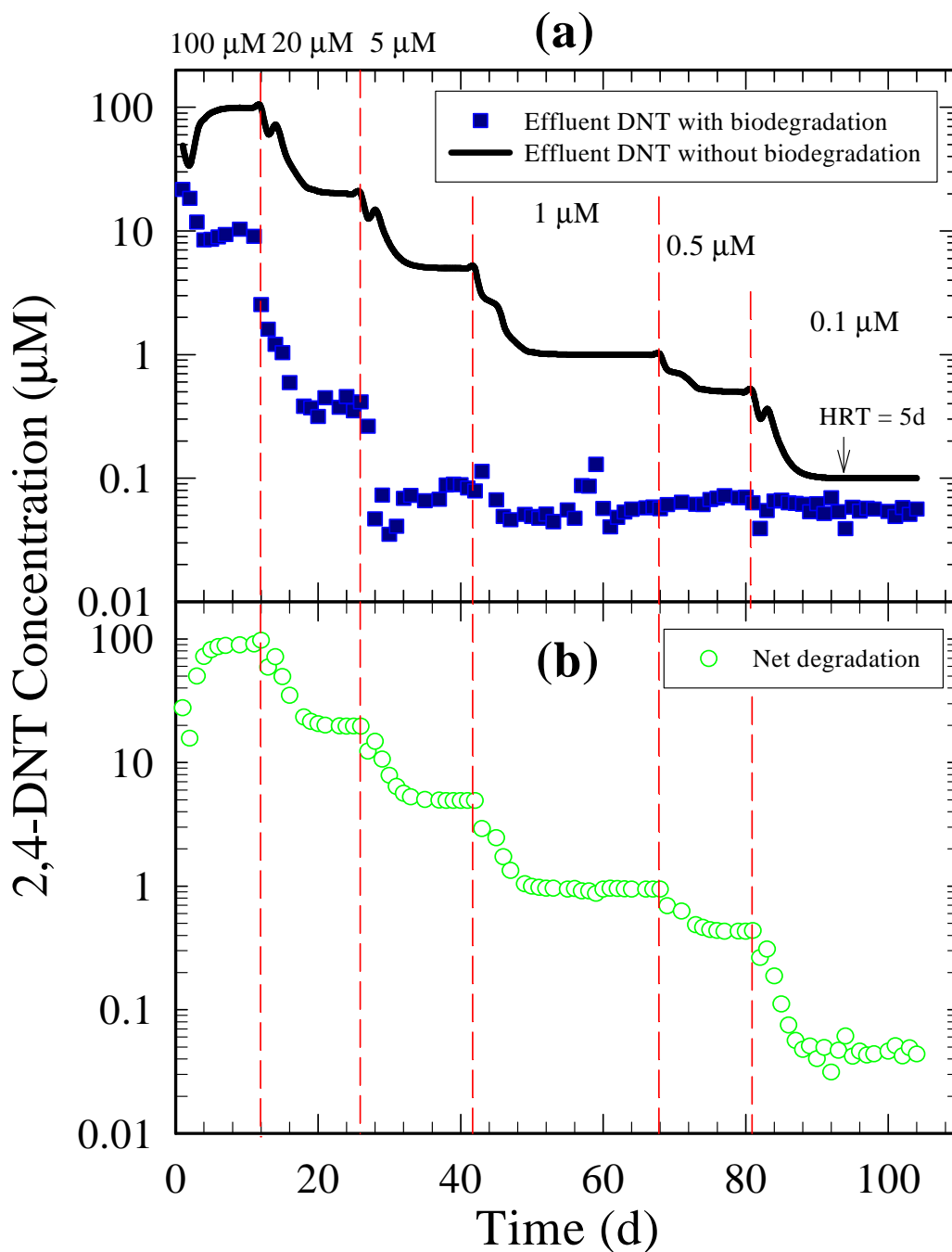
concentration of  $0.060 \pm 0.004 \mu\text{M}$  2,4-DNT. The flow rate was then reduced by a factor of 2, with an influent concentration of  $0.1 \mu\text{M}$  (*i.e.*, HRT was increased from 2.5 to 5 days) and no change in the effluent concentration were observed. As a result, this plateau value was considered to be the limiting value of 2,4-DNT degradation in the column system.

Effluent concentrations with no degradation were also predicted for the column system using one-dimensional transport models based on the convection-dispersion equation (CDE) (9).

$$R \frac{\partial S}{\partial t} = D \frac{\partial^2 S}{\partial x^2} - v \frac{\partial S}{\partial x} - K_r S \quad (6.5)$$

In the case of sorption-related transport,  $R$  is greater than 1.0. In this study, glass beads were used instead of soils to avoid adsorption that would complicate the assesement of aqueous phase DNT concentrations within the columns. In the absence of any sorption,  $R = 1.0$ . For conservative analysis  $K_r = 0$ , also. The other two column transport parameters ( $v$  and  $D$ ), based on parameters listed in Table 6.3, were evaluated as listed in Table 6.4 and each fit resulted in  $R^2$  values greater than 0.841. The calculated pore water velocities were comparable to the measured velocities and relatively low estimated  $D$  values were observed at the flow rates employed. The boundary and initial conditions are given by:

$$\frac{\partial S}{\partial t} = D \frac{\partial^2 S}{\partial x^2} - v \frac{\partial S}{\partial x} \quad (6.6)$$



**Figure 6.7.** (a) 2,4-DNT concentrations in column for influent concentrations of 100, 20, 5, 1, 0.5 and 0.1  $\mu\text{M}$  DNT. The solid line represents 2,4-DNT concentrations expected without biodegradation. (b) Actual 2,4-DNT degraded in column calculated by difference between experimentally measured and predicted values for 2,4-DNT concentration.



**Table 6.4.** Experimental and estimated transport parameter values for each column.

Column	Experimental values	Estimated values		
	Water pore velocity <sup>a</sup> ( $v$ , cm/min)	Water pore velocity ( $v$ , cm/min)	Dispersion coefficient ( $D$ , cm <sup>2</sup> /min)	$R^2$
A	0.0046	0.0038	0.0085	0.841
B	0.0042	0.0041	0.0072	0.985

\*A: 2,4-DNT degradation column, B: 2,6-DNT degradation column

<sup>a</sup> :  $v = Q$  (feed flow rate) divided by  $n$  (porosity)  $A$  (cross-sectional area)

$$\left\{ \begin{array}{l} \text{Initial Condition: } S(x, 0) = 0 \\ \text{Boundary Condition: } S(0, t) = C_0, \frac{\partial C}{\partial x}(x = \infty, t) = 0 \end{array} \right.$$

For these conditions, the analytical solutions (14) to Equation 6.6:

$$S(x, t) = \frac{S_0}{2} \left[ \operatorname{erfc} \left( \frac{x - vt}{2\sqrt{Dt}} \right) + \exp \left( \frac{vx}{D} \right) \operatorname{erfc} \left( \frac{x - vt}{2\sqrt{Dt}} \right) \right] \quad (6.7)$$

As a result, the breakthrough curves (BTCs) according for each influent concentration constructed based on this solution were determined and plotted (Figure 6.7a).

Based on difference between 2,4-DNT concentrations experimentally measured and predicted values in the column without biodegradation shown in Figure 6.7(a), the actual mass of 2,4-DNT degraded in the column was calculated as shown from Figure 6.7(b).

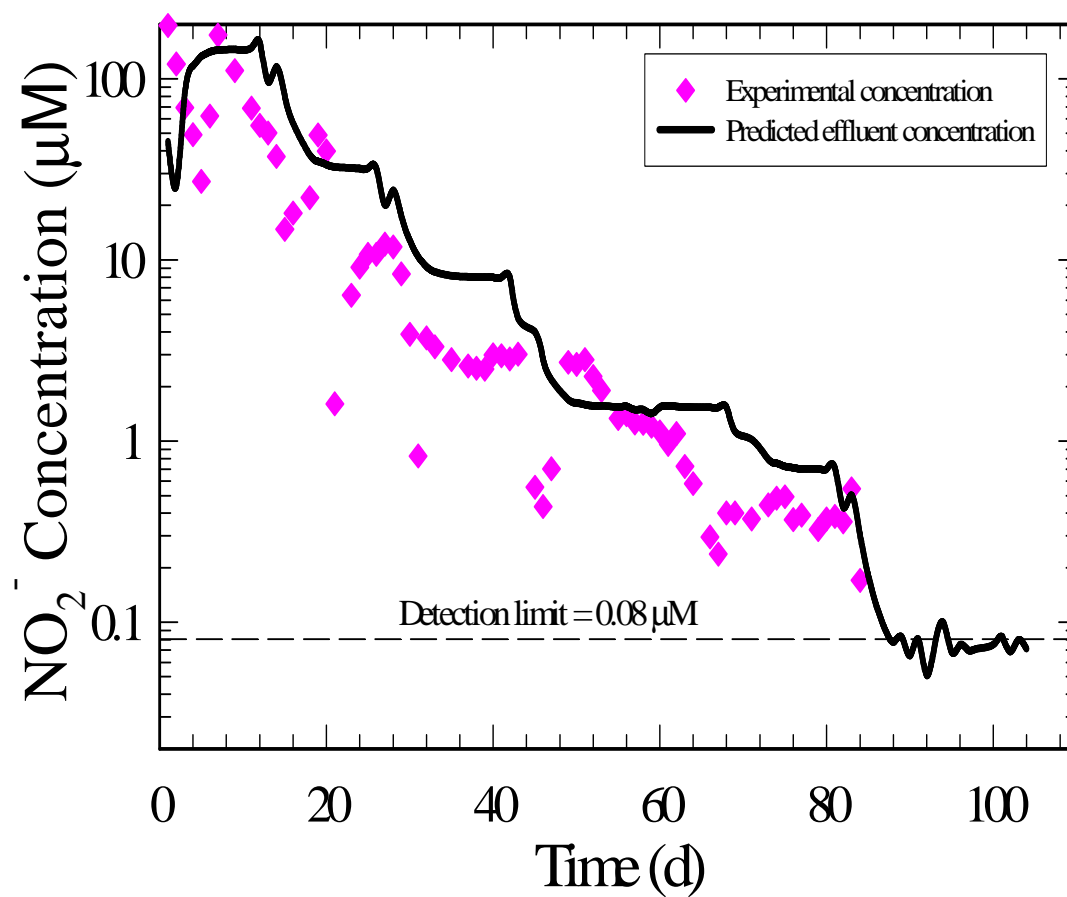
Biodegradation was immediately observed with an elevated influent of 100  $\mu\text{M}$  2,4-DNT, and corresponded with influent concentration reductions observed in the chemostats.

Data for nitrite concentrations in the column effluent are presented with effluent nitrite concentrations predicted by the mass of 2,4-DNT degraded and published stoichiometry (10) in Figure 6.8. Throughout the course of the experiment, nitrite eluded from the column at less than predicted concentrations, and was at levels below detection limits for the 0.1  $\mu\text{M}$  DNT influent concentration. Although the cause of lower than stoichiometric production of nitrite at low influent 2,4-DNT concentrations remains unknown, the effluent 2,4-DNT concentrations reached a limiting value (averaged at  $0.060 \pm 0.004 \mu\text{M}$ ) in the column system.

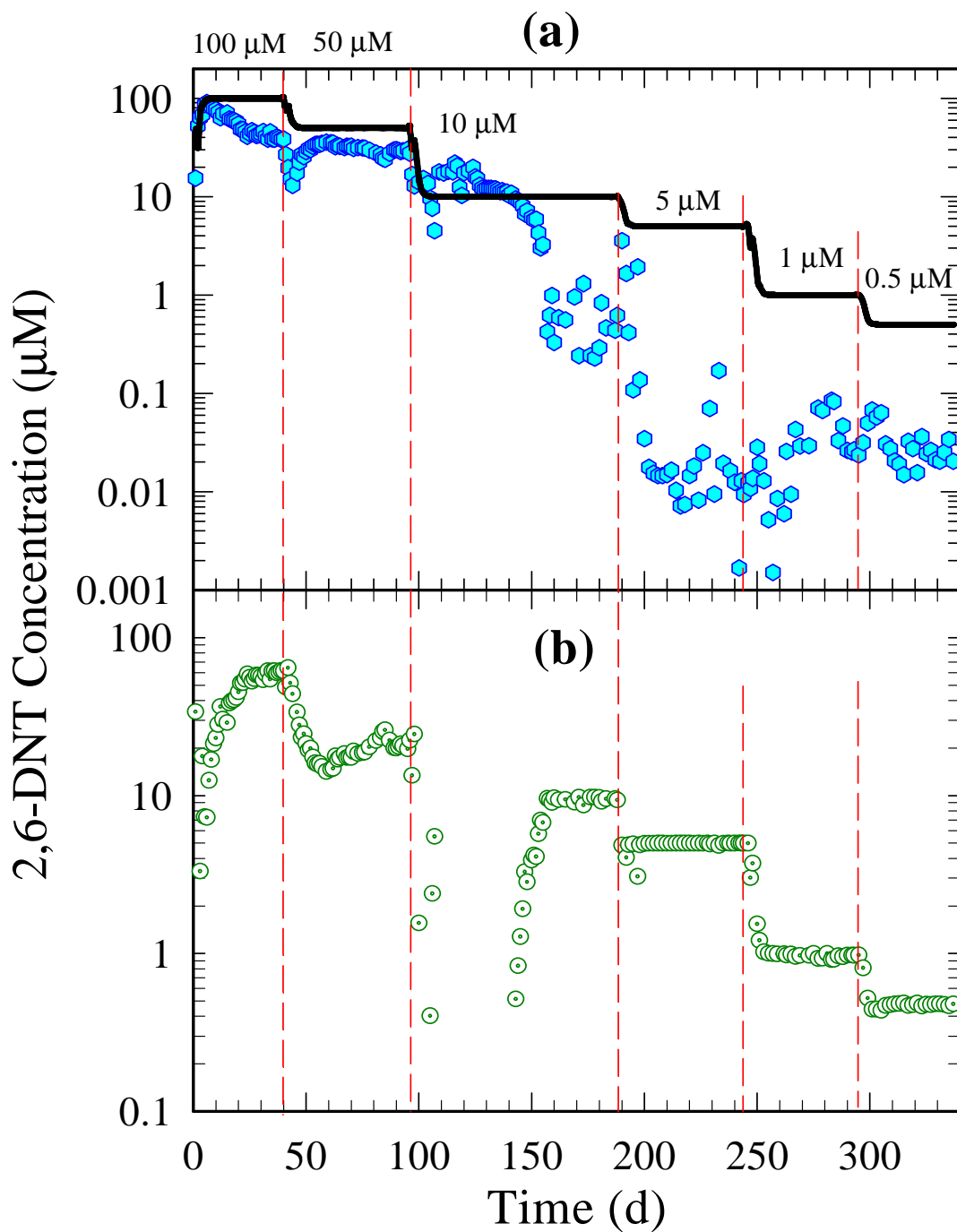
#### **6.4.2.2. 2,6-DNT Column Studies.**

In a similar manner, the limit of 2,6-DNT degradation was evaluated in a 2,6-DNT column after the inoculation with a 2,6-DNT degrading mixed culture. Figure 6.9a and 6.9b show the concentration of 2,6-DNT in the effluent at various influent concentrations and the net degraded 2,6-DNT, respectively. As 100  $\mu\text{M}$  of 2,6-DNT was fed into the column, an immediate drop in 2,6-DNT effluent concentrations were observed, reaching a steady state over approximately 25 days. The concentration of feed solution was then reduced to 50  $\mu\text{M}$ , where effluent concentration was stabilized after the reduction of 2,6-DNT in the effluent for the initial 30 days. Likewise, the influent level of 2,6-DNT was reduced gradually: 10, 5, 1, and 0.5  $\mu\text{M}$  steps. Steady-state concentrations of 2,6-DNT in the effluent were obtained ( $0.571 \pm 0.312$ ,  $0.023 \pm 0.006$ ,  $0.030 \pm 0.024$ , and  $0.025 \pm 0.006$   $\mu\text{M}$ ) at each feed of these concentrations, respectively, and no change was observed below an influent of 5  $\mu\text{M}$  (*i.e.*, limiting value for 2,6-DNT column averaged 0.026  $\mu\text{M}$ ). Mass balance calculations between 2,6-DNT removed and nitrite level produced in the column were performed and as presented in Figure 6.10, 2,6-DNT removal in the column was associated with the production near stoichiometric amounts of  $\text{NO}_2^-$ .

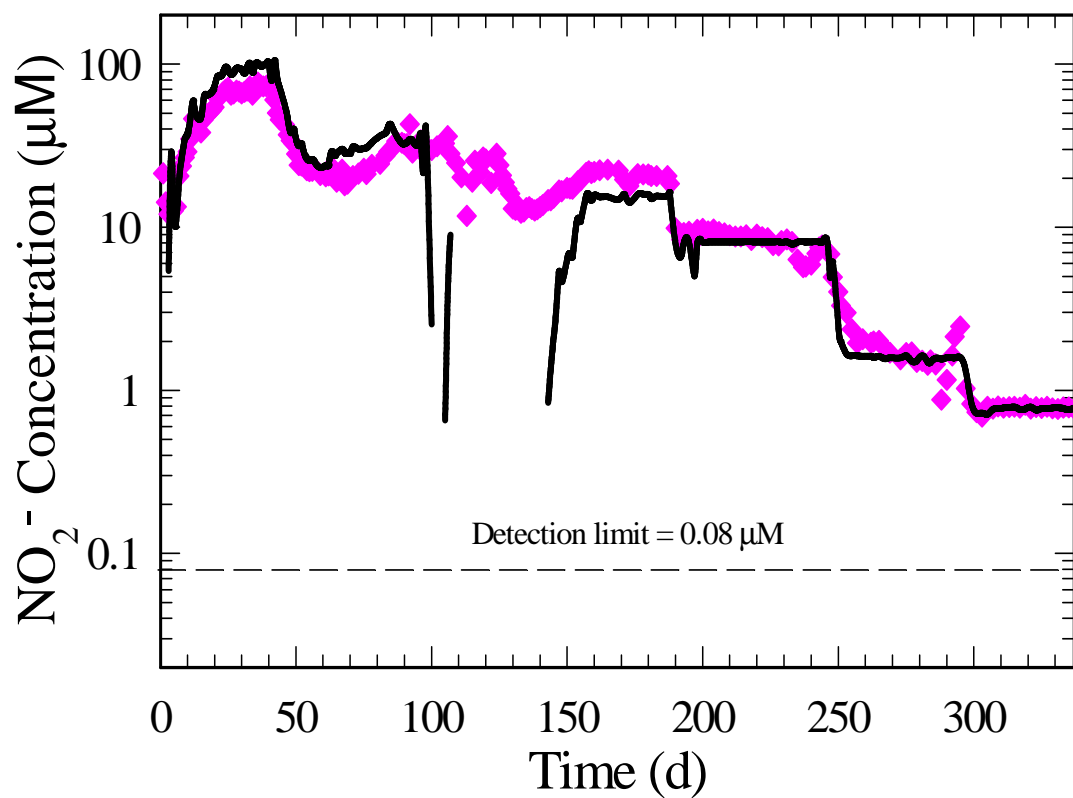
Commensurate with the nitrite production, a decrease in the pore water pH is anticipated, which can lead to cessation of degradation activity (5). It is reported that concentrations above 20 mM of nitrite and  $\geq 100$  mM are toxic to 2,4-DNT degrading strains and 2,6-DNT degrading strains, respectively (11) and DNT biodegradation can be inhibited at pH values of 6.0 and below (5). For both columns, effluent nitrite concentrations were below 10 mM, and effluent pH was generally 7.2 to 7.5. It is



**Figure 6.8.** Experimentally measured values for nitrite concentration in the column are presented with time. Expected values for nitrite concentration were calculated based on 2,4-DNT degraded in the column and stoichiometry provided by Zhang *et al.*(10).



**Figure 6.9.** (a) 2,6-DNT concentrations in column for influent concentrations of 100, 50, 10, 5, 1 and 0.5  $\mu\text{M}$  DNT. The solid line represents 2,6-DNT concentrations expected without biodegradation. (b) Actual 2,6-DNT degraded in column calculated by difference between experimentally measured and predicted values for 2,6-DNT concentration.



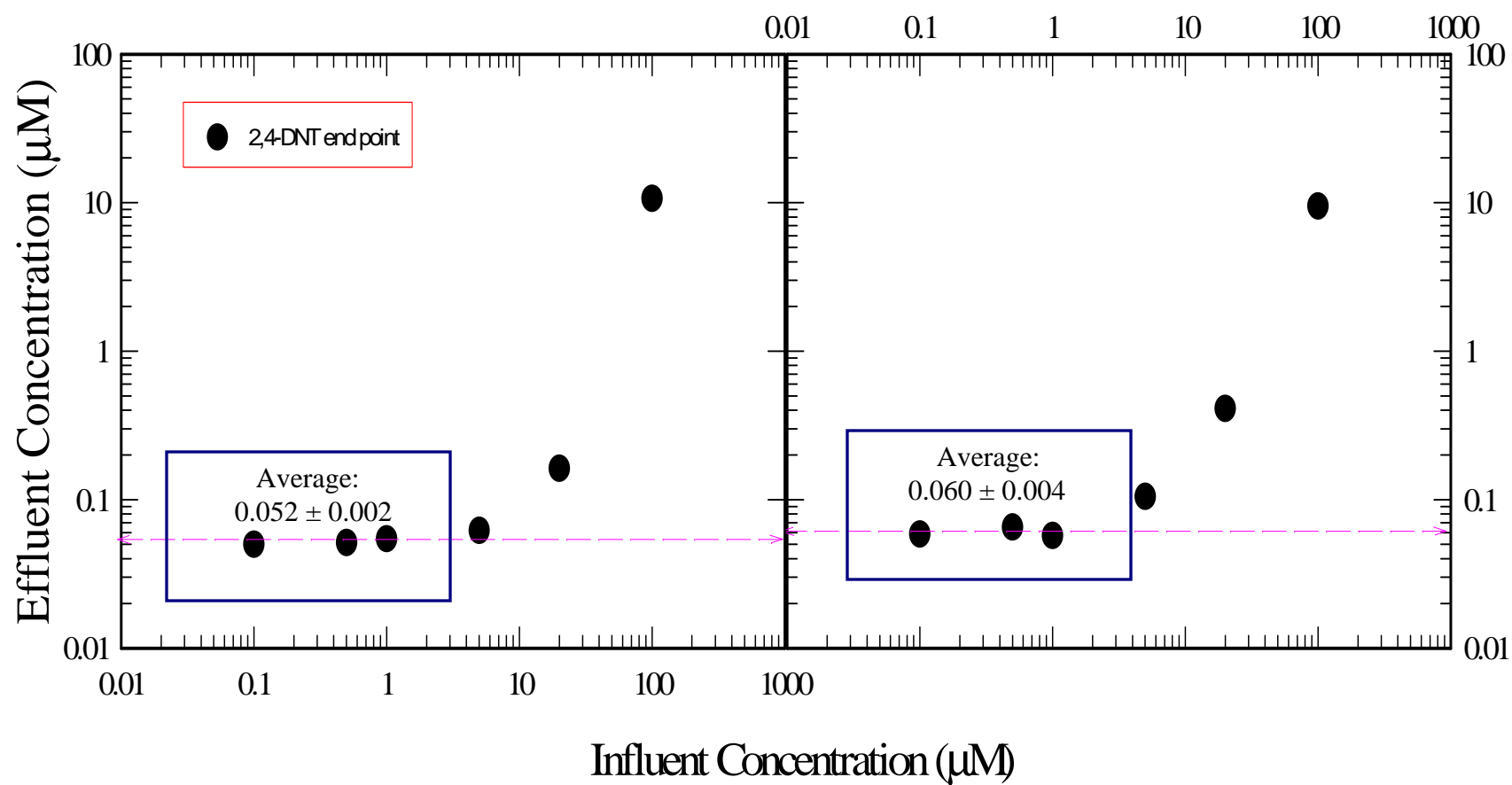
**Figure 6.10.** Experimentally measured values for nitrite concentration in the column are presented with time. Expected values for nitrite concentration were calculated based on 2,6-DNT degraded in the column and stoichiometry provided by Zhang *et al.*(10).

presumed that DNT degrading strains would be not inhibited by nitrite production and/or pH change associated with DNT degradation.

Dissolved oxygen (DO) concentration within columns is one of the factors which can also limit the complete biodegradation of DNT. Over the entire operation of columns, DO concentration in the feed solution was maintained at approximately 8 mg/L (*i.e.*, 250  $\mu\text{M}$   $\text{O}_2$ ) regardless of influent DNT concentration. Based on assumed stoichiometry ( $5.61:1 = \text{O}_2:\text{DNT}$  molar ratio) of the overall process described previously (10), 8 mg/L oxygen demand requires 44.6  $\mu\text{M}$  DNT degraded (*i.e.*,  $\Delta\text{DNT}/\Delta\text{O}_2 = 44.6/250$  molar base). Oxygen can be a limiting factor for degradation at high influent DNT concentrations (50 and 100  $\mu\text{M}$ ) if the only source of  $\text{O}_2$  is assumed to be the influent feed. Nevertheless, subsequent feed concentrations ( $\leq 20 \mu\text{M}$ ) of individual DNT isomers were below 8 mg/L oxygen demand thus ensuring available oxygen within columns.

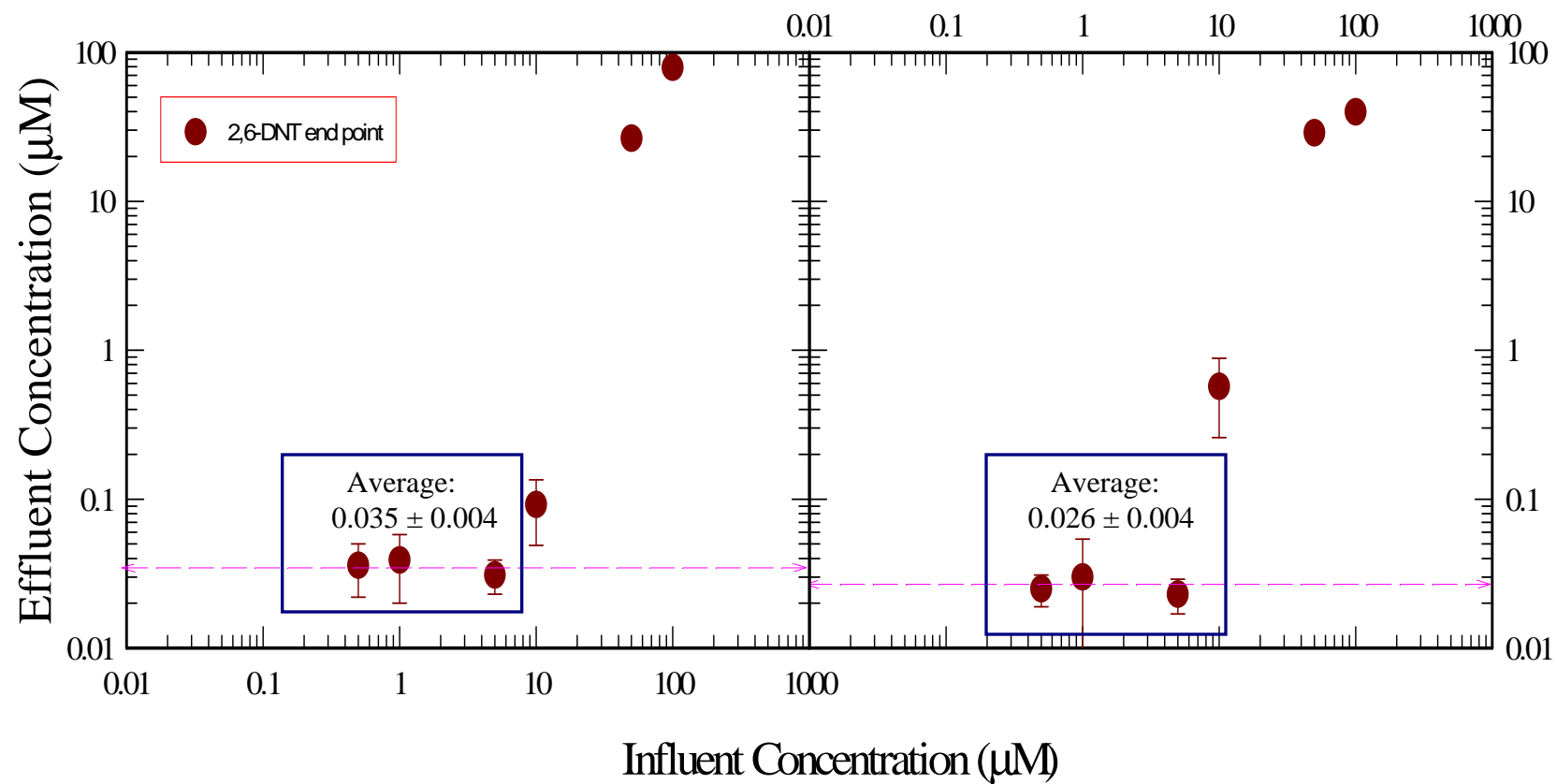
#### **6.4.3. Biodegradation Limits of DNT Isomers.**

To further investigate the minimum achievable DNT biodegradation limits for suspended and fixed film systems, values for steady-state DNT concentration in effluents were compared with influent DNT concentrations for column and chemostat systems. From the examination of data presented in Figure 6.11 and 6.12, steady-state effluent concentrations reach a plateau value at low influent values. The 2,4-DNT limits for chemostat and column systems averaged  $0.052 \pm 0.002 \mu\text{M}$  (6.11a) and  $0.060 \pm 0.004 \mu\text{M}$  (6.11b), respectively. The limiting value in the column was not statistically different from value observed in the chemostat at  $P \geq 0.05$ . The agreement between data for



**Figure 6.11.** Effluent 2,4-DNT concentrations at steady-state for various influent concentrations in the chemostat (a: Left) and column (b: Right) system. The plateau value for effluent concentration (biodegradation limit) is specified.





**Figure 6.12.** Effluent 2,6-DNT concentrations at steady-state for various influent concentrations in the chemostat (a: Left) and column (b: Right) system. The plateau value for effluent concentration (biodegradation limit) is specified.

column and chemostat systems provides strong evidence that at concentrations less than 0.052–0.060  $\mu\text{M}$ , 2,4-DNT can not support a sustained population of DNT degraders.

The same behavior was observed for 2,6-DNT. The minimum limiting value for the chemostat was  $0.035 \pm 0.004 \mu\text{M}$  (Figure 6.12a), and  $0.026 \pm 0.004 \mu\text{M}$  (Figure 6.12b) in the column system. As estimated beforehand, 2,6-DNT experiments displayed lower plateau values than from the 2,4-DNT experiments.

Daprato *et al.* (1) used a mixture culture taken from a same inoculum source as employed herein. Based on 2,4DNT biodegradation kinetic parameters obtained from regression of batch-fed slurry reactor studies calculated values of  $S_{\min}$  (Table 6.1) are higher than the limiting values determined in the current study. Consequently, predictions of  $S_{\min}$  may vary within mixed cultures as a function of enrichment conditions. In spite of such differences, biodegradation limits reported in this chapter were directly obtained experimentally and came from systems operated under conditions more closely related to those applicable to NA when DNT concentrations are low.

#### **6.4.4. Natural Attenuation Potential for DNTs.**

Regulatory criteria for 2,4-DNT and 2,6-DNT in drinking water in the United States were presented in Table 6.2. Important regulatory values for consideration include the drinking water criteria level provided by the US EPA (6) for a risk factor of less than  $10^{-6}$  and contaminant levels required for industrial waste streams outlined by the Code of Federal Regulations (CFR). Limiting biodegradation concentrations for 2,4-DNT (*i.e.*,  $10.93 \pm 0.73 \mu\text{g/L}$  for column and  $9.47 \pm 0.36 \mu\text{g/L}$  for chemostat) and 2,6-DNT (*i.e.*,  $4.74 \pm 0.73 \mu\text{g/L}$  for column and  $6.37 \pm 0.73 \mu\text{g/L}$  for chemostat) experimentally

measured in this study were lower than the values recommended by the US EPA and the CFR, suggesting that NA based plume remedies that include DNT mineralization may achieve site specific treatment goals, assuming that oxygen and other nutrient requirements are available. However, the complexity of the natural systems derives the extensive studies, such as estimation of biodegradation limits in the presence of various cosubstrates (15).

## 6.5. References

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## CHAPTER 7

### Cometabolic Biotransformation of Trinitrotoluene Supported by Dinitrotoluenes under Aerobic Conditions

#### 7.1. Overview

Two DNT degrading mixed cultures, one fed 2,4-DNT and the other fed 2,6-DNT, were enriched from soil samples contaminated with munitions waste. Cultures were exposed to TNT in batch and chemostat studies to evaluate TNT degradation, and the effect of TNT on DNT consumption in the presence of DNT. The 2,4-DNT degrading culture removed 2,4-DNT in batch and chemostat systems and also consumed TNT. The culture enriched with 2,6-DNT was inhibited by the presence of TNT. The threshold value for TNT that inhibited 2,6-DNT degradation ranged from 15 to 33  $\mu\text{M}$  in this particular culture. When 2,6-DNT degradation occurred, the degradation of TNT was observed to varying degrees.

To examine the fate of transformed TNT, radiolabeled tracer tests were conducted in microcosms using the 2,4-DNT culture to determine the fate of TNT-derived metabolite products (*i.e.*,  $\text{CO}_2$ , particulate, and aqueous  $^{14}\text{C}$ ). These experiments showed minor production of  $^{14}\text{CO}_2$  and small fraction of  $^{14}\text{C}$  associated with particulate after 35 d of incubation while TNT was completely transformed. Intermediates identified included an oxidative metabolite (*i.e.*, TNT derived catechol on the basis of retention time and UV spectra published) and other unknown metabolites. The majority of transformed TNT was associated with polar products that were not identified by HPLC analysis.

## 7.2. Introduction

To date, microorganisms capable of growth on TNT as a primary energy or carbon source have not been identified, and only limited TNT mineralization has been observed in diverse biological system (1-3). As a result, *in situ* TNT bioremediation analogous to DNT bioremediation is not believed to be feasible and active remediation (e.g., ozone-ultraviolet light treatment, composting, and incineration) is a presumed remedy (4-9). At TNT manufacturing sites there are areas contaminated with mixtures of TNT and DNT isomers. Interestingly, this mixture of contaminants may serve to promote *in situ* bioremediation of TNT. Research conducted under the direction of Dr. Spain at the AFRL (Air Force Research Laboratory) has shown that bacteria capable of growth on DNT can carry out the transformation of TNT while degrading DNT (10) and it is possible that at sites where DNT/TNT mixtures are present that this process is ongoing. The extent to which TNT disappearance at these locations can be attributed to cometabolism, or how to incorporate this process into the assessment of TNT fate is unknown. If the extent of TNT cometabolism during DNT degradation can be defined, it may be possible to estimate the loss of TNT caused by DNT degraders, and include this mechanism in evaluations of *in situ* bioremediation at these sites.

To investigate the effect of DNT on TNT cometabolism by DNT degrading cultures, batch experiments were conducted with various ratios of either 2,4-DNT or 2,6-DNT to TNT. Chemostats were also operated at varying influent 2,4-DNT/TNT and 2,6-DNT/TNT ratios to create systems with a range of net degradation activity and to determine the extent of TNT cometabolism proportional to DNT degradation.

Additionally,  $^{14}\text{C}$ -TNT was employed in closed microcosms containing effluent from chemostats to conduct mass balances, including  $^{14}\text{CO}_2$  production (11).

### **7.3. Materials and Methods**

#### **7.3.1. Chemicals.**

2,4,6-trinitrotoluene (TNT  $\geq 99\%$ ) was purchased from Chem Service (West Chester, PA). The standard chemicals for identification of intermediates (*i.e.*, ADNTs, and diaminonitrotoluenes) were obtained from Dr. Ronald Spanggord of SRI International and Dr. Spain. and  $^{14}\text{C}$ -TNT was supplied by Dr. Spain (originally purchased from American Radiolabelled Chemicals, Inc). The radiochemical purity of  $\geq 98\%$  was determined using HPLC. The radioisotope  $^{14}\text{C}$ -TNT (50  $\mu\text{Ci/mL}$ ) was dissolved in 50 % ethanol. The  $^{14}\text{C}$ -TNT was added at the concentration of approximately 40,000 dpm/L (*i.e.*, 4,000 dpm/100ml and 0.018  $\mu\text{Ci/mL}$ ) in experimental systems.

#### **7.3.2. Experimental Cultures.**

Cultures employed in this experiment included a 2,4-DNT degrading mixed culture and a 2,6-DNT degrading mixed culture (Sources: Badger Army Ammunition Plant, WI for 2,4-DNT and Tyndall Air Force Base, FL for 2,6-DNT) used in Chapter 4. The cultures have been maintained in a continuously stirred, draw and fill reactor in the laboratory for over 3 years.

#### **7.3.3. Analytical Methods.**



ZORBAX SB-C18 column was used for the separation of DNT and TNT (13). The other Hybercarb® porous graphite column (100 x 3 mm, 5µm, Thermo Hypersil, UK) was used for the separation between DNT isomers (14). The Standard Methods for measurement of nitrite and nitrate were described in previous Chapters (15).

#### **7.3.4. Batch Studies.**

Samples of enrichment cultures (10 mL of 2,4-DNT and 50 mL of 2,6-DNT degrading mixed culture) were transferred to flasks (150 mL) containing mineral media (100 mL and 50 mL respectively) with a single DNT isomer, TNT alone, or in a DNT/TNT mixture. Once the culture media was formulated and inoculated, batch reactors were mixed and incubated at 22°C. Batch reactors containing TNT were covered with aluminum foil to prevent photo-degradation of TNT, and all microcosms were maintained on a stir plate at 1,500 rpm.

#### **7.3.5. Continuous Stirred Tank Reactor (CSTR) Studies.**

Two CSTRs, fed individual DNT isomers, were operated to assess the potential for sustained cometabolism of TNT. The schematics of CSTR systems were described in Chapter 6. The 2,4-DNT/TNT-CSTR was inoculated with the 2,4-DNT degrading mixed culture and was fed 200 mL media daily with a 2.5 d HRT. The 2,6-DNT/TNT-CSTR was inoculated with the 2,6-DNT degrading mixed culture and was fed 100 mL media daily with a 5 d HRT. For both reactors, the working volume was 500 mL, and the feed concentrations of DNT and TNT are presented in Table 7.1. Reactor influent was

**Table 7.1.** Chemostat studies: Influent composition and time line.

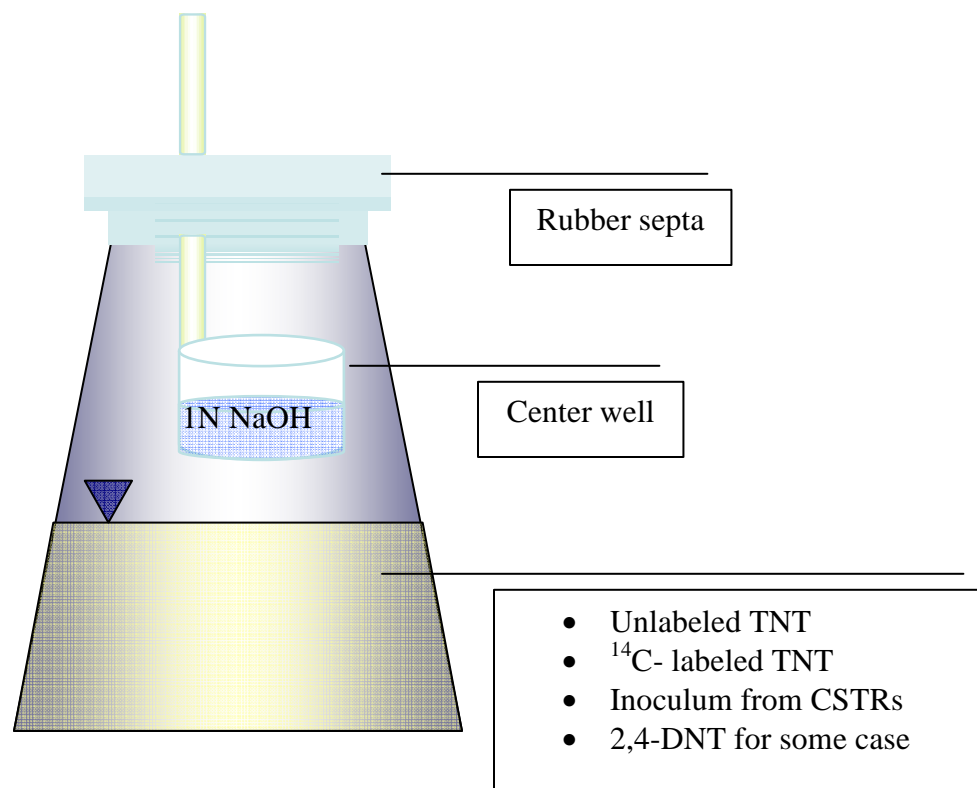
Chemostats <sup>a</sup>	Running Time (days)	Ratio of influent compositions 2,4- or 2,6-DNT / TNT ( $\mu\text{M}$ )
A	0 – 40	$111 \pm 5.2 / 18 \pm 0.8$
	41 – 58	$226 \pm 16 / 18 \pm 0.8$
	59 – 94	$226 \pm 16 / 37 \pm 0.6$
	95 – 124	$226 \pm 16 / 67 \pm 1.5$
	125 – 155	$226 \pm 16 / 100 \pm 0.7$
	156 – 187	$226 \pm 16 / 141 \pm 2.7$
	188 – 227	$0 / 141 \pm 2.7$
B	0 – 59	$81 \pm 4.1 / 15 \pm 1.1$
	60 – 107	$169 \pm 10 / 15 \pm 1.1$
	108 – 144	$169 \pm 10 / 33 \pm 1.4$
	145 – 193	$288 \pm 0.5 / 33 \pm 1.4$
	194 – 227	$288 \pm 0.5 / 71$
<sup>a</sup> Chemostat Id: A, 2,4-DNT/TNT-CSTR; B, 2,6-DNT/TNT-CSTR with low feed concentration of TNT		

provided by a syringe pump, and outflow was hydraulically controlled to maintain a constant volume. Both CSTRs were operated at varying influent DNT concentration to create systems with varying net degradation activity. After systems reached steady state DNT consumption, TNT was introduced to the feed solution and was monitored for TNT disappearance. After TNT disappearance reached steady state, the ratio of concentrations (*i.e.*, DNT and TNT) in the influent was altered and monitoring was continued.

#### ***7.3.6. Radioactive Tracer Experimentation.***

Inoculum (25 mL) from the CSTR fed 2,4-DNT was amended with TNT at a concentration of approximately 44  $\mu\text{M}$  (10 mg/L) and 5  $\mu\text{L}$   $^{14}\text{C}$ -TNT stock solution (50  $\mu\text{Ci/mL}$  dissolved in 50 % ethanol, > 99 % pure TNT). This solution was stirred for 30 min to ensure dissolution of  $^{14}\text{C}$ -labeled TNT. Next, 5 mL of this solution was transferred to flasks (10 mL) fitted with rubber septa and center well (containing 1.0N NaOH) that penetrated the stopper of the reactor (Figure 7.1).

In a separate experiment, TNT mineralization was examined with 2,4-DNT (200  $\mu\text{M}$ ) as a cosubstrate. 2,4-DNT (36 mg) was added to an empty reactor and CSTR effluents, containing inocula and TNT, were added and stirred for 1 hr until it was completely dissolved. Next,  $^{14}\text{C}$ -TNT (5  $\mu\text{L}$ ) was added in these solutions (25 mL), and was stirred for 30 min. Active reactors (with TNT alone and with both TNT and 2,4-DNT) and sodium-azide inhibited control (200 mg/L  $\text{NaN}_3$  and 10  $\mu\text{M}$   $\text{HgCl}_2$ ) were run in duplicate to demonstrate repeatability of results.



**Figure 7.1.** Schematic diagram of batch reactor for  $^{14}\text{C}$  tracer studies.

#### **7.3.6.1. Sample Collection and Analysis.**

The mass of media in reactors was recorded at each sampling event and mass balance calculations were based on these measurements. Each sample event recorded 3 fractions: aqueous  $^{14}\text{C}$  containing TNT and metabolites;  $^{14}\text{C}$  associated with particulate material; and,  $^{14}\text{CO}_2$ . Each of these fractions was mixed with scintillation cocktail and analyzed by liquid scintillation cocktail (LSC).

##### ***7.3.6.1.1. Monitoring of Gaseous $^{14}\text{CO}_2$ Evolution ( $^{14}\text{CO}_2$ ).***

$^{14}\text{CO}_2$  was trapped in the center well containing 1.0 N NaOH. Periodically, the  $\text{CO}_2$  trap (100  $\mu\text{L}$ ) was withdrawn, and the well was rinsed with water (100  $\mu\text{L}$ ) again to extract  $^{14}\text{CO}_2$  remained. These aqueous samples (total 200  $\mu\text{L}$ ) were added into 2.5 mL scintillation cocktail containing 500  $\mu\text{L}$  MeOH. The radioactivity was analyzed in LSC.

##### ***7.3.6.1.2. Radioactivity in Aqueous TNT and Metabolites (Aqueous $^{14}\text{C}$ ).***

Samples (100  $\mu\text{L}$ ) were recovered from the each reactor and centrifuged for 5 min. Supernatant was mixed with cocktail (2.5 mL) in scintillation vial. Centrifuged pellet was rinsed with MeOH (100  $\mu\text{L}$ ) to extract soluble  $^{14}\text{C}$ , and recentrifuged. Supernatant combined with previous supernatant and cocktail in scintillation vial. The radioactivity of the combined supernatants was analyzed in LSC.

##### ***7.3.6.1.3. Radioactivity Associated with Particulate ( $^{14}\text{C}$ -Particulate).***

The debris that remained after centrifugation was rinsed with MeOH (100  $\mu$ L). After shaking, the suspension placed in a scintillation vial with 2.5 ml of cocktail. These samples were counted with the LSC to determine the particulate associated radioactivity.

#### **7.3.6.1.4. Radioactivity of Residual $^{14}\text{CO}_2$ within Aqueous $^{14}\text{C}$ .**

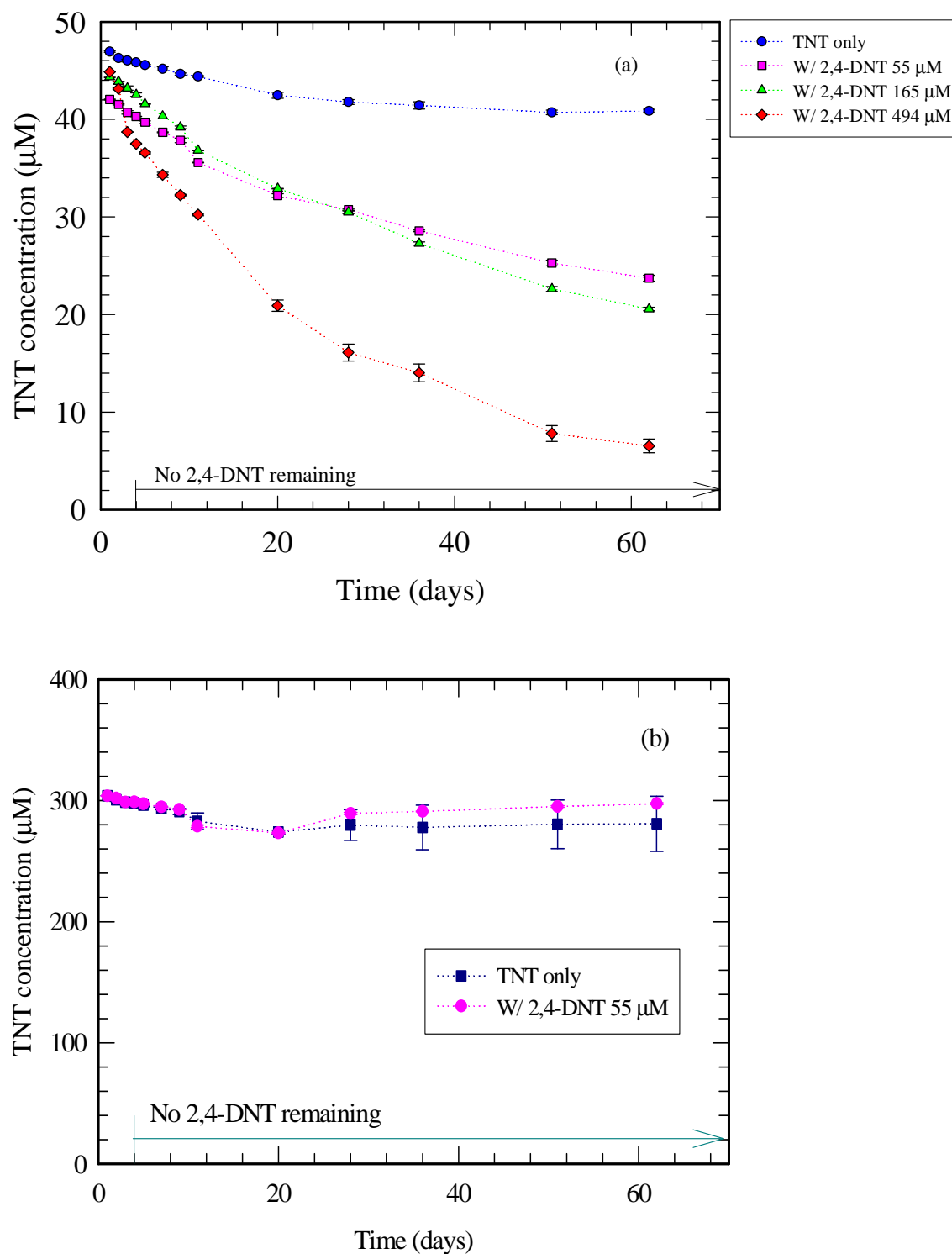
At the end of the study reactors were acidified with 2.5 % acetic acid to trap dissolved  $^{14}\text{CO}_2$ , and counted to determine quantity of  $^{14}\text{C}$  that was volatile after acidification. Same behavior presented above was conducted to estimate radioactivity for each fraction.

HPLC analysis of metabolites and TNT was conducted with a Varian HPLC system equipped with a diode array detector. Acidified samples (1 ml) were recovered from each vial and centrifuged. Supernatant (100  $\mu$ L) was analyzed with HPLC. The fraction containing metabolites was eluted within 20 min and each fraction was divided before and after 20 sec. The resulting fractions were separately collected in scintillation vials and mixed with 2.5 ml of cocktail.

## **7.4. Results and Discussion**

### **7.4.1. Batch Studies.**

Data shown in Figure 7.2 present the degradation of TNT by the 2,4-DNT enrichment culture. The 2,4-DNT unamended control (Figure 7.2A) demonstrated little or no TNT removal. In DNT amended systems, aqueous TNT was disappeared. TNT degradation with 494  $\mu\text{M}$  2,4-DNT was more rapid and to a higher extent than in systems with lower 2,4-DNT additions. After 60 days of incubation, the removal of TNT was 44,

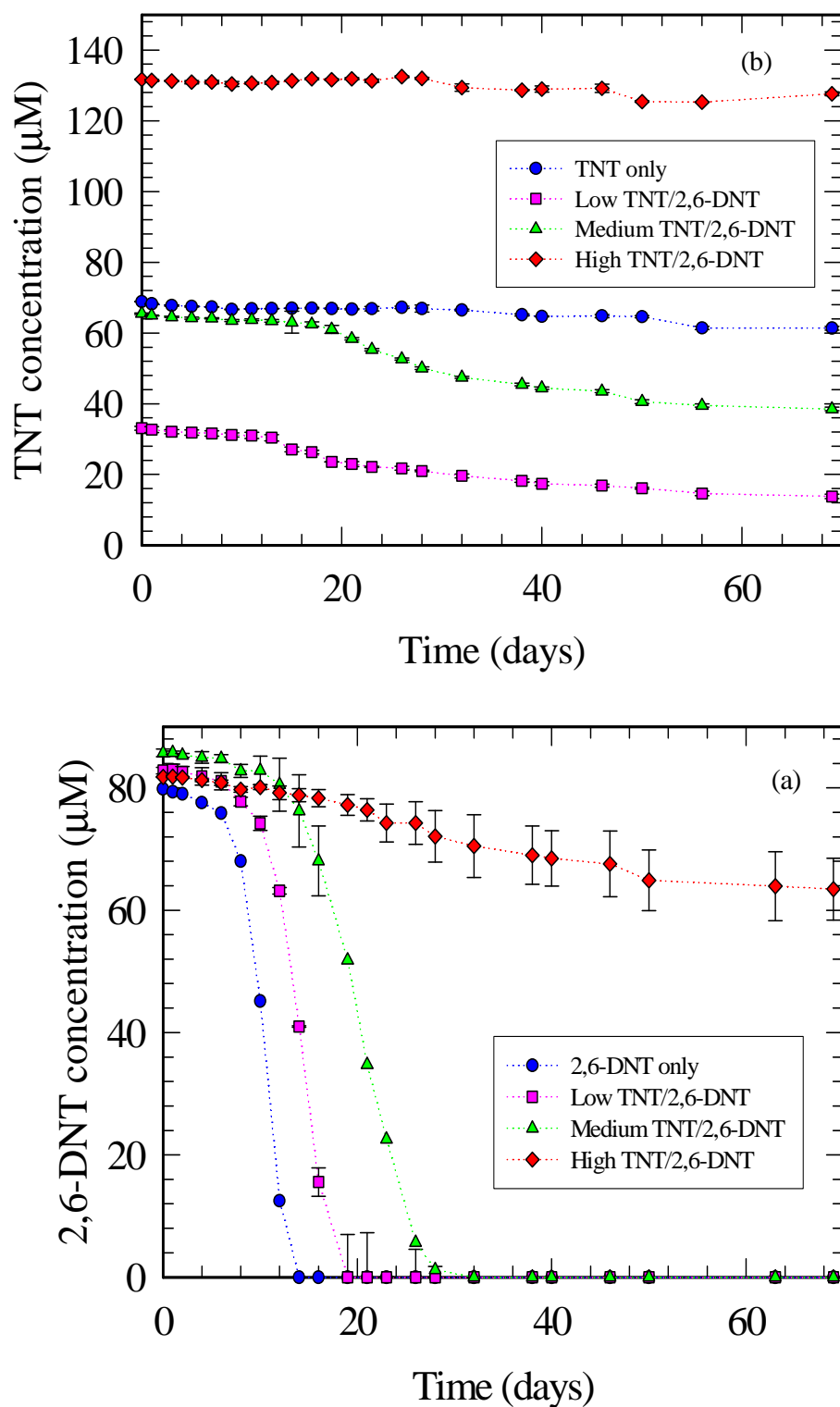


**Figure 7.2.** Data for a batch reactor with exposure of 2,4-DNT degrading organisms to 2,4-DNT in the presence of (a) low TNT concentration and (b) high TNT concentration. Note: each 2,4-DNT was completely removed in an system within 5 days.

54, and 85 % at 2,4-DNT concentrations of 55, 165, and 494  $\mu\text{M}$  2,4-DNT, respectively. Batch reactors fed high concentration of TNT (304  $\mu\text{M}$ ) and 55  $\mu\text{M}$  2,4-DNT were conducted and results are shown in Figure 7.2 (b). Little to no TNT decrease was observed, suggesting that high concentrations of TNT may cause inhibition of TNT (co-)metabolism in 2,4-DNT degrading culture. Along with TNT degradation, the degradation of 2,4-DNT was monitored. 2,4-DNT in all reactors was completely removed within 5 d, indicating that the rate of 2,4-DNT degradation was not inhibited by the presence of TNT.

In parallel experiments, the disappearance of TNT in mixture with 2,6-DNT was assessed using the 2,6-DNT enrichment culture. Results indicated that TNT was metabolized when 2,6-DNT was biodegraded. However, the rate and extent of 2,6-DNT degradation were influenced by increasing TNT concentrations. Figure 7.3 (a) shows that even low levels of TNT resulted in slower 2,6-DNT degradation, and inhibition became severely limiting at 132  $\mu\text{M}$  TNT. Figure 7.3 (b) presents the concentration of TNT over time at varying ratio of TNT and 2,6-DNT. The 2,6-DNT unamended control and the system containing 132  $\mu\text{M}$  TNT revealed little to no removal of TNT. The other mixed cultures with both TNT and 2,6-DNT demonstrated minimal removal of TNT concentration during incubation. These observations suggest that 2,6-DNT degradation in the presence of TNT will be slower than when present alone, and that the loss of TNT due to 2,6-DNT degradation will likely be minimal.





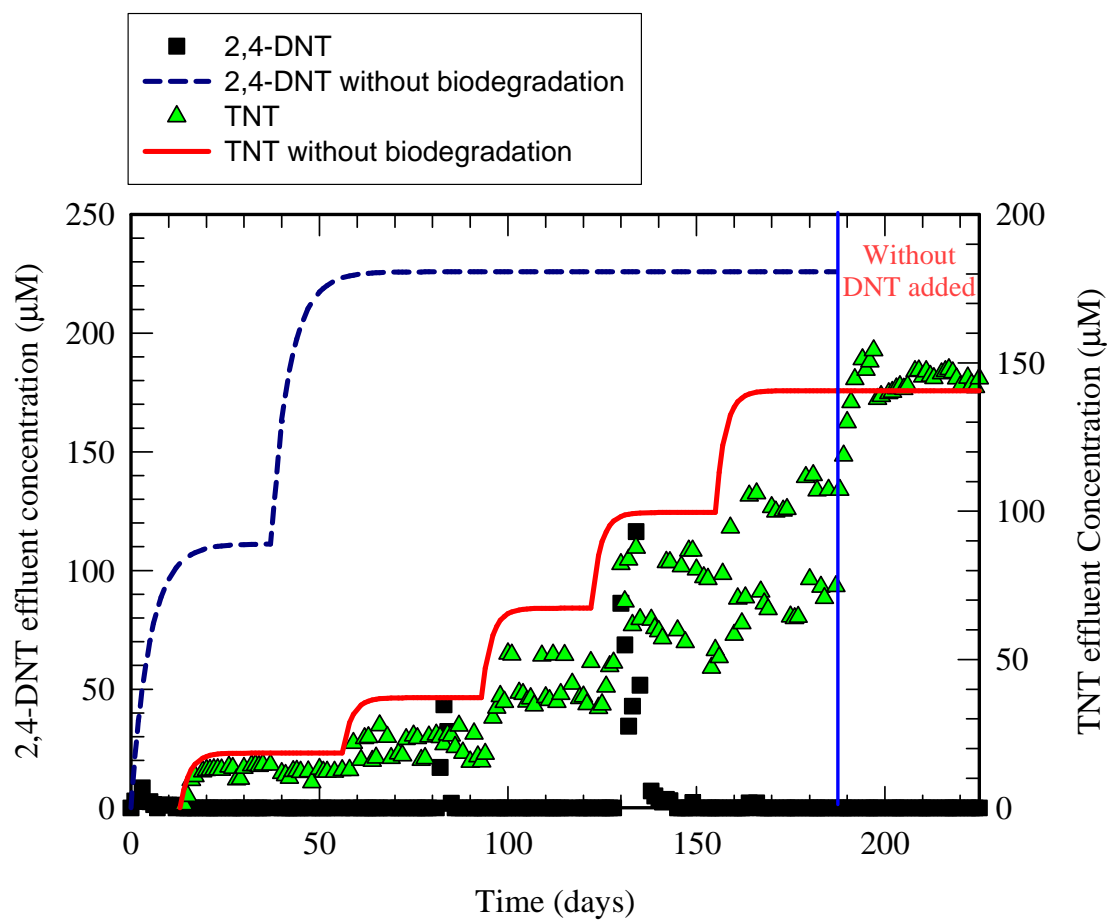
**Figure 7.3.** Data for a batch reactor with exposure of 2,6-DNTdegrading organisms to 2,6-DNT in the presence of TNT; (a) 2,6-DNT concentration (b) TNT concentration over time.

#### 7.4.2. CSTR Studies.

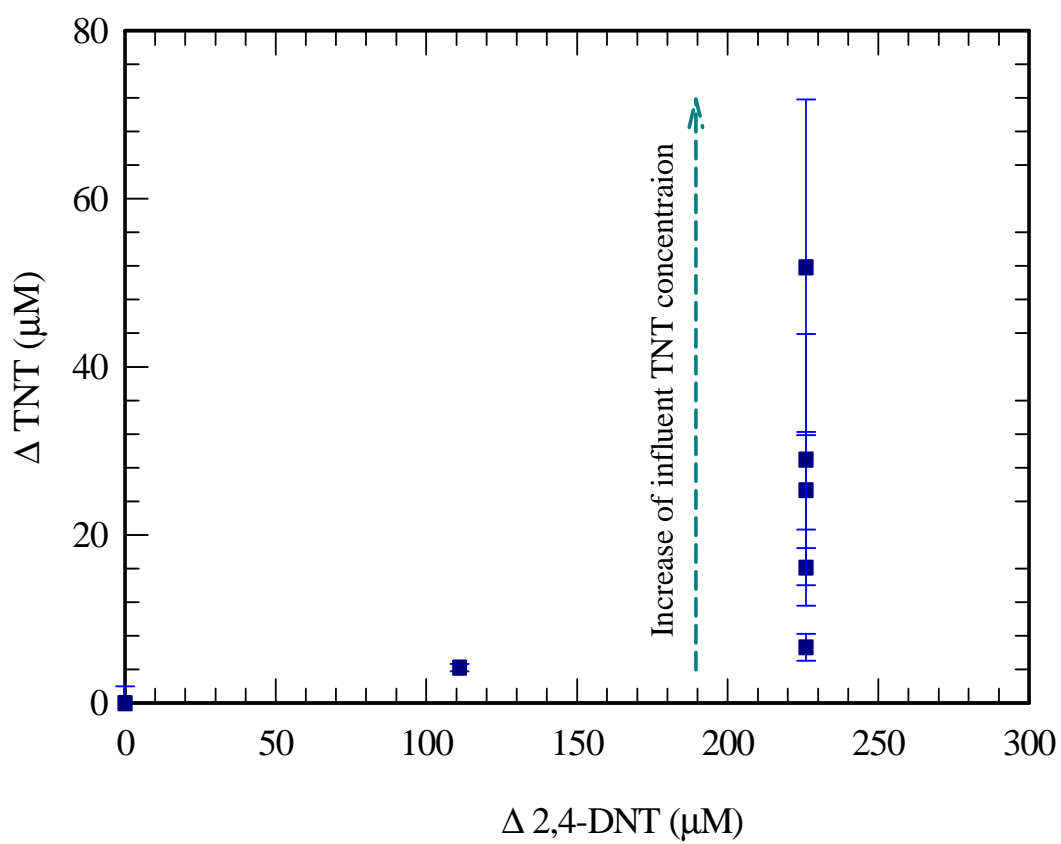
CSTRs were operated to examine the extent of TNT cometabolism relative to DNT degradation in continuous culture. Results of the 2,4-DNT/TNT CSTR and 2,6-DNT/TNT CSTR are presented in Figure 7.4 and 7.6, respectively. In these Figures, results of sample analysis are presented in comparison to the DNT and TNT concentrations in the reactors if no degradation was occurring. As presented in Chapter 6, the concentration profiles of DNT and TNT were predicted without considering biodegradation under non-steady state condition using equation 6.4.

In Figure 7.4 when 2,4-DNT alone ( $111 \pm 5.2 \mu\text{M}$ ) fed to CSTR, 2,4-DNT effluent concentration was near detection limits ( $0.1 \mu\text{M}$ ). After approximately 10 retention times, a low concentration of TNT ( $18 \pm 0.8 \mu\text{M}$ ) was introduced to the feed solution. Steady-state TNT effluent concentration was  $14 \pm 0.4 \mu\text{M}$ . On day 40 2,4-DNT influent concentration increased to  $226 \pm 16 \mu\text{M}$ , and steady-state TNT effluent concentration decreased to  $12 \pm 1.6 \mu\text{M}$ . At the constant 2,4-DNT concentration in feed solutions, TNT influent concentration increased in step, and correspondingly increase of steady-state TNT effluent concentration was observed at  $21 \pm 4.5$ ,  $42 \pm 6.9$ ,  $70.6 \pm 15$ , and  $89 \pm 20 \mu\text{M}$  respectively. After no addition of 2,4-DNT to CSTR on day 188, TNT effluent concentration rose rapidly near values ( $141 \pm 2.7 \mu\text{M}$ ) expected without biodegradation. Complete degradation of 2,4-DNT was observed in all samples, with only a minor perturbation at day 82 and 130.

To evaluate the ratio of TNT cometabolism attributable to 2,4-DNT degradation activity, the amount of TNT removed ( $\Delta \text{TNT}$ ) was plotted against the amount of 2,4-DNT removed ( $\Delta \text{2,4-DNT}$ ) as presented in Figure 7.5. The net degradation (*i.e.*  $\Delta \text{TNT}$



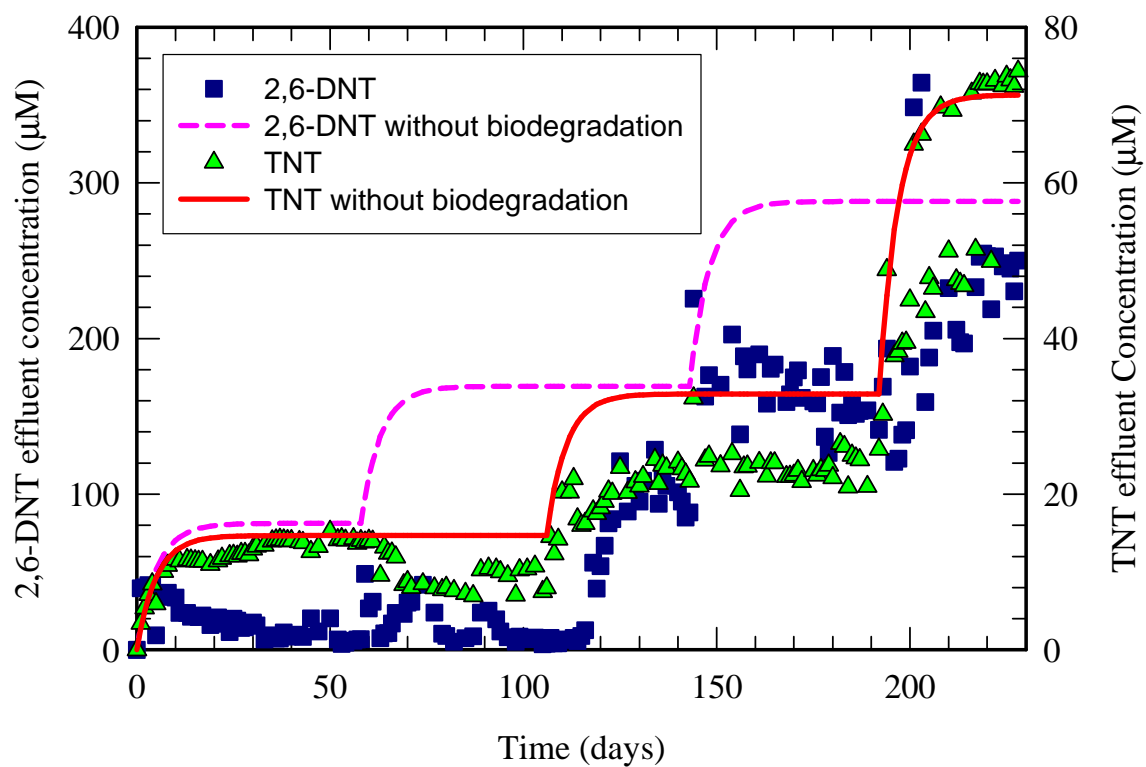
**Figure 7.4.** 2,4-DNT and TNT concentrations in 2,4-DNT/TNT mixed contaminant CSTR. Expected reactor concentrations based on influent concentration without biodegradation are provided (dash and solid lines).



**Figure.7.5.** Estimation of TNT cometabolism for 2,4-DNT degradation in 2,4-DNT/TNT mixture based on results from CSTR studies.

and  $\Delta$  2,4-DNT) was determined by the difference between predicted concentration (dash or solid line) and measured values (data points) at steady state. TNT was metabolized non-stoichiometrically during 2,4-DNT degradation. Initially, 2,4-DNT was degraded ( $111 \pm 5.2 \mu\text{M}$  as  $\Delta$  2,4-DNT) with low TNT degradation ( $4.2 \pm 0.4 \mu\text{M}$  as  $\Delta$  TNT). The possibility of abiotic transformation or adsorption of TNT to biomass can not be ruled out, and the actual quantity of TNT disappeared was uncertain. Complete 2,4-DNT removal remains at higher 2,4-DNT influent concentration ( $226 \pm 16 \mu\text{M}$  as  $\Delta$  2,4-DNT), and a slightly higher TNT removal was observed ( $6.6 \pm 1.6 \mu\text{M}$  as  $\Delta$  TNT). However, this is not statistically different from initial net degradation of TNT at  $P \geq 0.05$ . TNT concentration in feed solution increased in stepwise manner as mentioned above, and steady-state TNT net degradation increased to  $16 \pm 4.5$ ,  $25 \pm 6.9$ ,  $29 \pm 15$ , and  $52 \pm 20 \mu\text{M}$  respectively. On average higher TNT was removed under constant 2,4-DNT degradation conditions, but variation in data was too high to prove statistically significant. There was no growth on TNT independent of 2,4-DNT because 2,4-DNT effluent concentration reached the values predicted with biodegradation when no 2,4-DNT was added to reactor on day 188.

The experimental protocol for determination of TNT cometabolism for 2,6-DNT degrading mixed cultures paralleled that outlined for 2,4-DNT/TNT CSTR. Figure 7.6 presents the degradation profiles over time at varying ratios of TNT to 2,6-DNT fed to the CSTR. Degradation activity was initiated with a concentrations of 2,6-DNT and TNT at  $81 \pm 4.1 \mu\text{M}$  and  $15 \pm 1.1 \mu\text{M}$ , respectively. The steady-state 2,6-DNT effluent concentration was  $8.3 \pm 5.4 \mu\text{M}$  and experimentally measured TNT concentrations were at the values that would be expected without no degradation in CSTR. On day 59, 2,6-

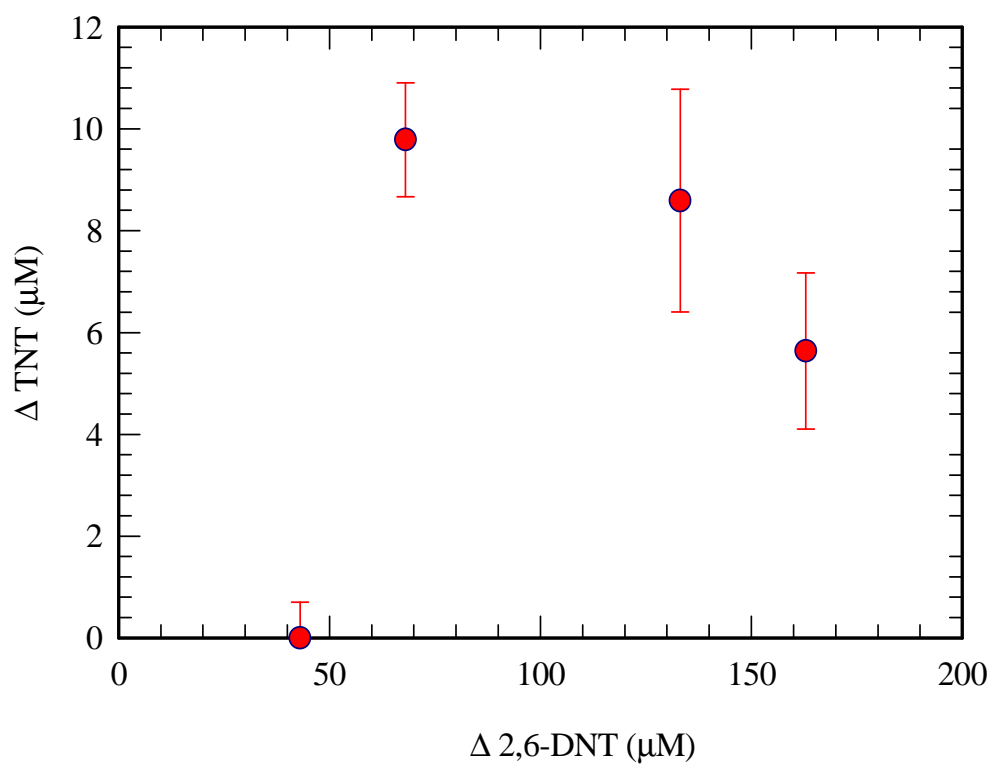


**Figure 7.6.** 2,6-DNT and TNT effluent concentrations in the CSTRs with varying ratios of 2,6-DNT and TNT. Expected reactor concentrations based on influent concentration without biodegradation are provided (dash and solid lines).

DNT influent concentration increased to  $169 \pm 10 \mu\text{M}$ , which resulted in the immediate decrease of effluent TNT concentration where steady-state TNT effluent concentration was  $9.0 \pm 1.5 \mu\text{M}$ . Subsequently when TNT concentration in feed solution increased to  $33 \pm 1.4 \mu\text{M}$ , the steady-state TNT effluent concentration was  $23 \pm 1.1 \mu\text{M}$ , but the rapid increase of 2,6-DNT in the effluent was observed to  $101 \pm 14 \mu\text{M}$ . The 2,6-DNT in feed solution then decreased to  $288 \pm 0.5 \mu\text{M}$  2,6-DNT to possibly increase TNT metabolism. No significant change of TNT effluent concentration was observed ( $24 \pm 2.2 \mu\text{M}$  at steady state), rather an elevation of 2,6-DNT level (averaging  $155 \pm 11 \mu\text{M}$ ). At the final stage (increase of TNT in the feed solution,  $71 \mu\text{M}$ ) TNT effluent concentration reached near expected value with a significant increase of 2,6-DNT concentration in the effluent ( $245 \pm 12 \mu\text{M}$ ). These observations indicate that 2,6-DNT degrading mixed cultures metabolized TNT during 2,6-DNT degradation, but the high concentration of TNT inhibited both 2,6-DNT degradation and TNT cometabolism processes.

Initially, 2,6-DNT was removed with no measurable degradation of TNT over the first 58 days. Thus, the net degradation of TNT vs. 2,6-DNT ( $\Delta \text{TNT}$  vs.  $\Delta \text{2,6-DNT}$ ) except this period was estimated as conducted at 2,4-DNT/TNT CSTR (Figure 7.7). From day 60 to day 192, as the amount of 2,6-DNT removed increased, the amount of TNT removed decreased. During the last 35 days operation, small amount of 2,4-DNT was degraded with no TNT degradation. This indicates that TNT was metabolized during 2,6-DNT degradation at lower, less TNT inhibitory levels.

To further assess TNT cometabolism in the presence of each DNT isomer, nitrite concentration in the reactor was monitored and compared with anticipated nitrite release (dash line) based on the stoichiometry described by Zhang *et al.*, (1 mole of DNT yields



**Figure 7.7.** Estimation of TNT cometabolism for 2,6-DNT degradation in 2,6-DNT/TNT mixture based on results from CSTR studies.



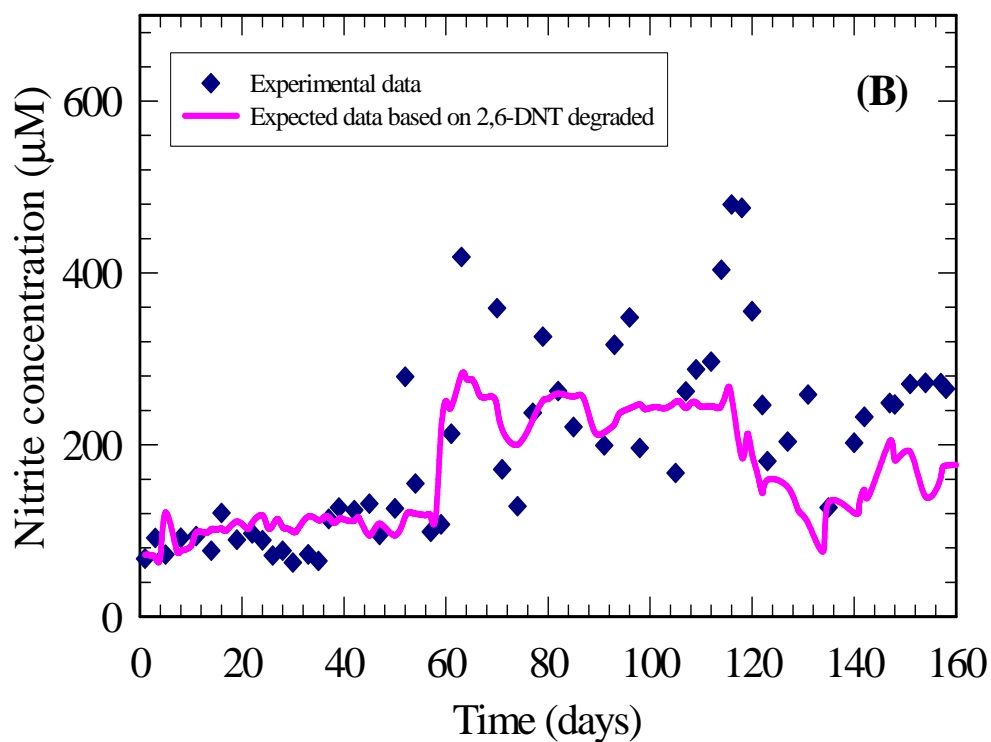
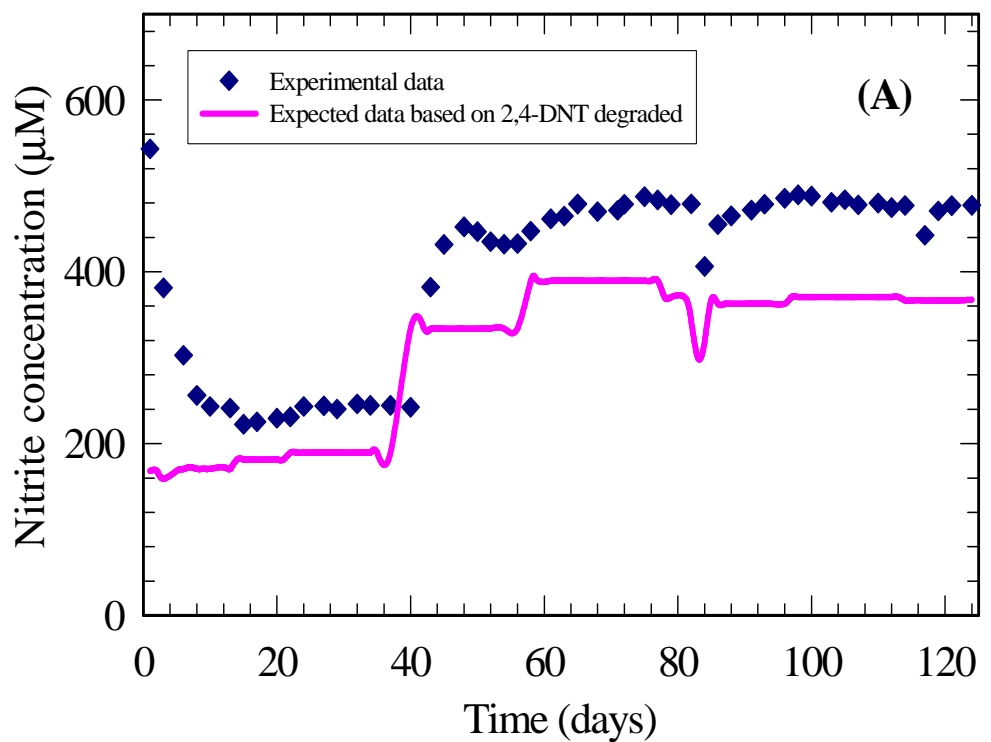
1.63 moles of nitrite) (Figure 7.8A) (17, 18). Interestingly, the observed release of nitrite was more than predicted over the entire operation.  $\Delta \text{NO}_2$  (the difference of dash line and data points) was  $54 \pm 2.3$ ,  $106 \pm 9.1$ ,  $92 \pm 28$ , and  $100 \pm 28 \mu\text{M}$  at steady state, indicating nitrite production from different source (*i.e.*, TNT metabolism). Despite non-stoichiometric release of nitrite, a high level of nitrite in the reactor can be attributed to both 2,4-DNT and TNT degradation.

Data from nitrite analysis in 2,6-DNT/TNT CSTR are presented with effluent nitrite concentrations predicted by the mass of 2,6-DNT degraded and published stoichiometry (Figure 7.8B). Throughout the course of the experiment, nitrite levels were similar to or slightly higher than predicted stoichiometric quantities. At the first stage, production near stoichiometric amount of nitrite was observed to parallel 2,6-DNT degradation in the reactor. On day 59 (at the beginning of significant TNT removal), nitrite levels began to increase, and mostly were higher than stoichiometrically derived values. It is possible that the transformation of TNT may be due to oxidative microbial degradation of TNT through the removal of nitro group.

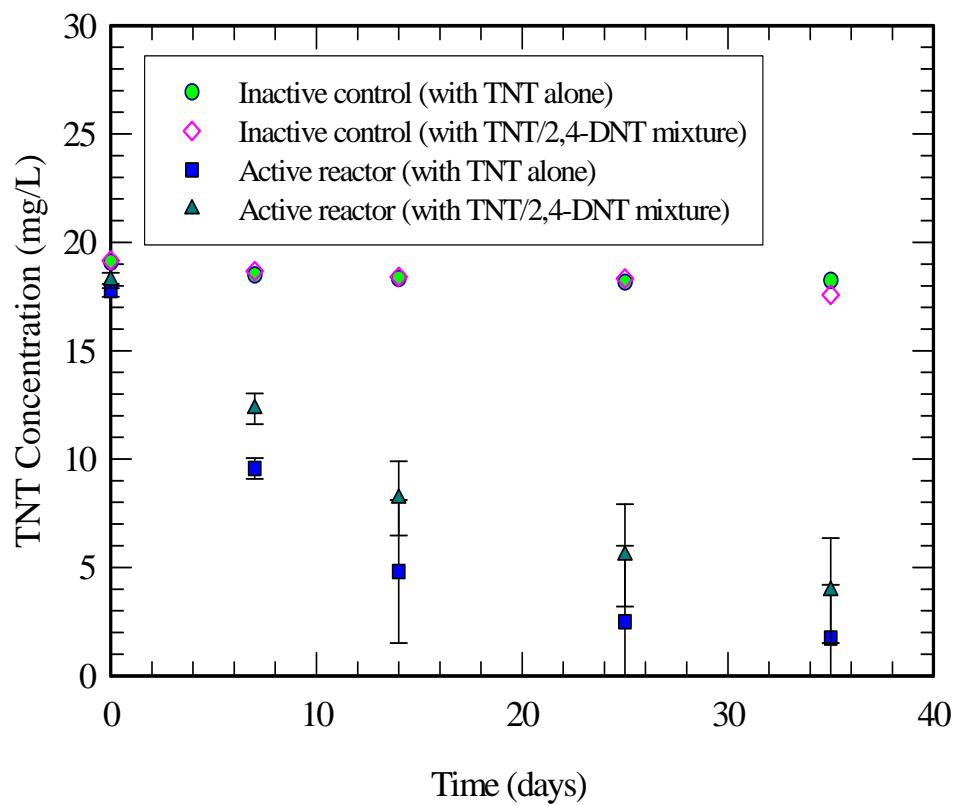
### **7.4.3. <sup>14</sup>C Tracer Studies.**

#### **7.4.3.1. <sup>14</sup>C distribution of <sup>14</sup>C-TNT fed alone and in TNT/2,4-DNT mixtures.**

Results of <sup>14</sup>C tracer studies are presented in Figure 7.9. TNT in active reactors was depleted without a discernible lag period, and depletion was nearly complete within 35 d of incubation. Negligible losses of TNT were observed in sodium-azide inhibited controls. The first order rates of TNT depletion were estimated for active systems; when TNT was applied to microcosms individually, it was depleted at the maximal rate of 0.86



**Figure 7.8.** Experimentally measured values for nitrite concentration in CSTR are presented with time. Expected values for nitrite concentration were calculated based on (A) 2,4-DNT and (B) 2,6-DNT degraded and stoichiometry provided by Zhang *et al.*(18).

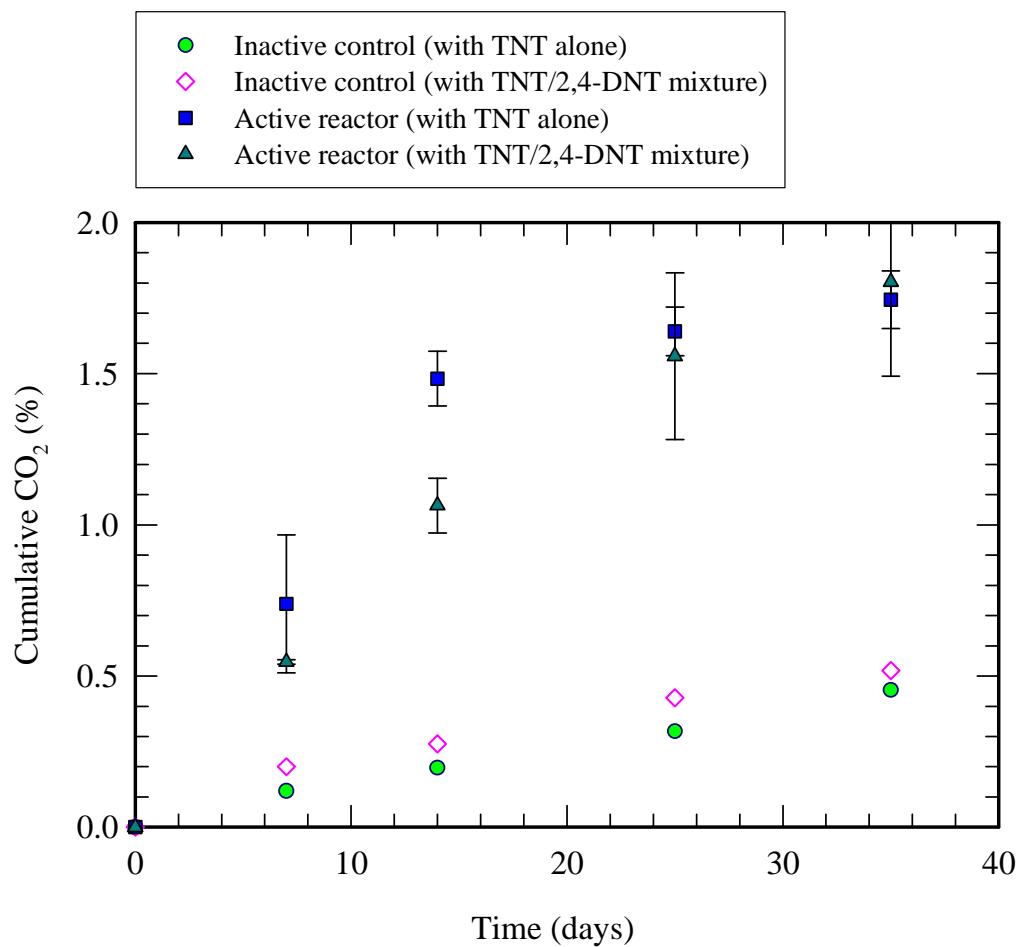


**Figure 7.9.** Batch studies: TNT degradation shown by inoculum obtained from CSTR. Each system contained TNT alone and TNT/2,4-DNT mixtures as carbon and nitrogen sources.

$\pm 0.0086 \text{ mg/L}\cdot\text{d}$  ( $R^2 = 99 \%$ ). When present in TNT/2,4-DNT mixtures, the estimated rate of TNT depletion was at  $0.049 \pm 0.0038 \text{ mg/L}\cdot\text{d}$  ( $R^2 = 99 \%$ ), which was slower than the alone TNT depletion rate. Overall the degradation of TNT was not observed to occur rapidly, and additional analysis was needed to understand the product distribution.

The distribution of  $^{14}\text{C}$  was investigated and products of TNT were characterized with periodic HPLC samples. Cumulative  $^{14}\text{CO}_2$  over time are presented in Figure 7.10. Despite considerable removal of TNT after 35 days of incubation, evolution of  $^{14}\text{CO}_2$  was insignificant in active reactors. The maximal rates of mineralization were estimated at  $0.50 \%$   $^{14}\text{CO}_2/\text{d}$  (Logarithm,  $R^2 = 96 \%$ ) and  $0.45 \%$   $^{14}\text{CO}_2/\text{d}$  (Logarithm,  $R^2 = 92 \%$ ), in active reactors with TNT alone and TNT/2,4-DNT mixture, respectively.

The final distribution of  $^{14}\text{C}$  radioactivity is listed in Table 7.2, where data for total  $^{14}\text{C}$ , cumulative  $^{14}\text{CO}_2$  production,  $^{14}\text{C}$  associated with particulate and aqueous  $^{14}\text{C}$  are shown at the end of the experiments. A low amount of  $^{14}\text{C}$ -TNT was mineralized;  $2.2 \pm 0.3 \%$  of  $^{14}\text{C}$  in active reactors with TNT alone. In addition to low production of  $^{14}\text{CO}_2$ , low incorporation of  $^{14}\text{C}$  to particulate coincided with high fraction of aqueous  $^{14}\text{C}$ . In inactive control, negligible amounts of aqueous  $^{14}\text{C}$  were depleted to possibly the sampling process and no appreciable  $^{14}\text{CO}_2$  was evolved. When applied as 2,4-DNT/TNT mixtures, the overall distribution of  $^{14}\text{C}$  was similar to the TNT alone experiment. Data from control reactor indicated low  $^{14}\text{C}$ -TNT conversion to  $\text{CO}_2$  ( $0.61 \%$ ) after 35 days incubation.



**Figure 7.10.** Percentage mineralization of  $^{14}\text{C}$ -TNT to  $^{14}\text{CO}_2$  over 35 day incubation in inactive and active microcosms.

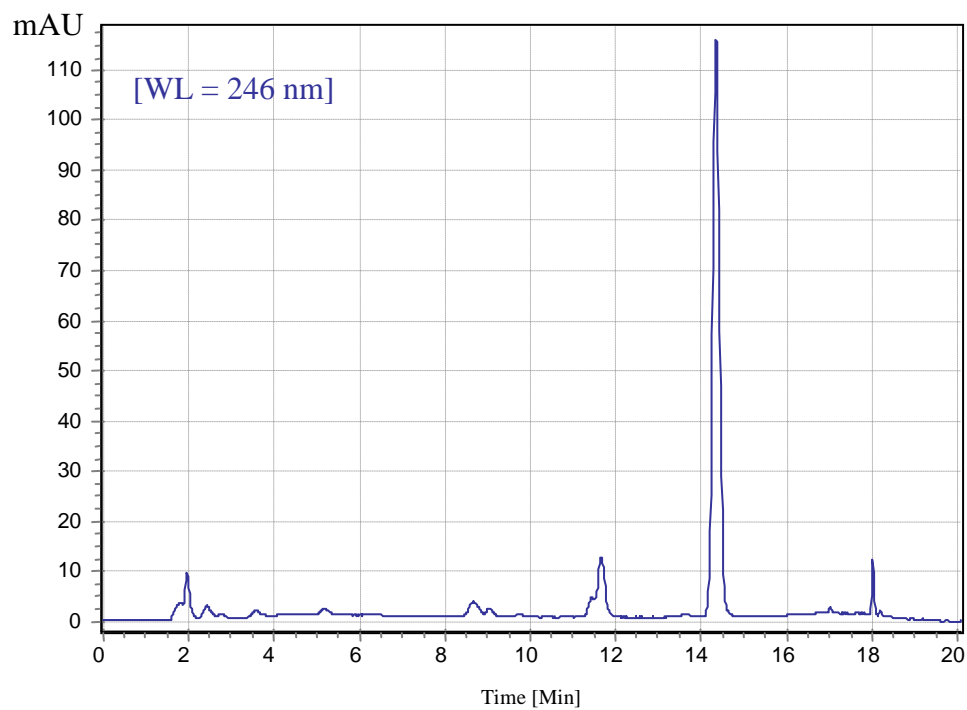
**Table 7.2.** Final distribution of  $^{14}\text{C}$  radioactivity in active microcosms with TNT alone and 2,4-DNT/TNT mixture after 35 d.

		Radioactivity (%)			
		Total $^{14}\text{C}$	$^{14}\text{CO}_2$	Aqueous $^{14}\text{C}$	$^{14}\text{C}$ -particulate
TNT alone	Active	$82 \pm 1.4$	$2.2 \pm 0.3$	$75 \pm 2.8$	$3.7 \pm 0.6$
	Killed Control	96	0.5	97	2.0
2,4-DNT/ TNT mixture	Active	$88 \pm 5.5$	$2.3 \pm 0.2$	$85 \pm 8.3$	$3.7 \pm 1.1$
	Killed Control	96	0.6	98	1.3

#### **7.4.3.2. Fate of Biotransformed TNT – Characterization of Metabolites.**

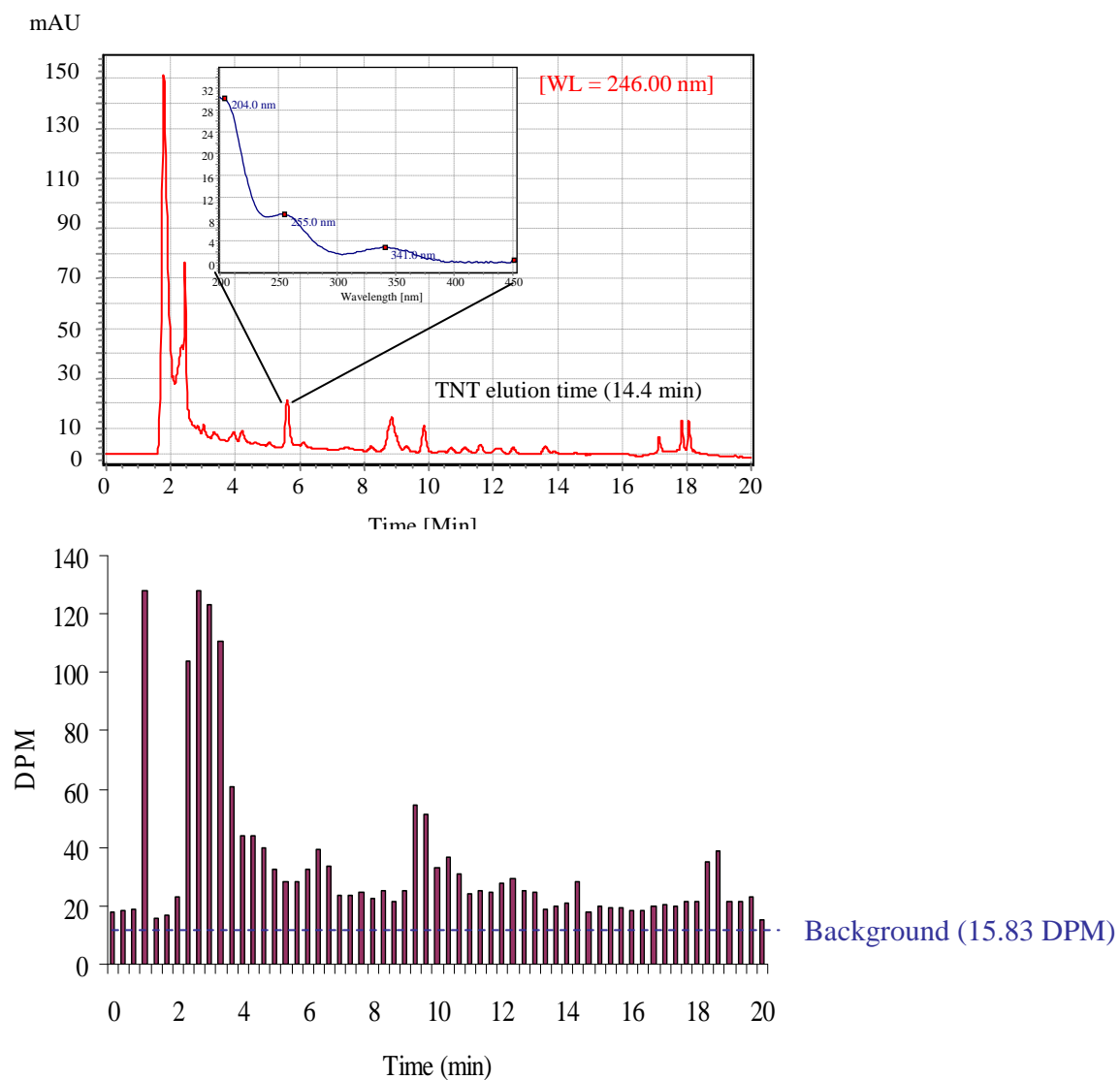
To further examine the fate of  $^{14}\text{C}$ -TNT, aqueous  $^{14}\text{C}$  in reactor was separated and fractionated via HPLC. TNT loss and certain metabolites were identified using external standards (TNT, 2,4-DNT, 2,6-DNT, 2-amino-4,6-DNT, 4-amino-2,6-DNT, and 2,4-Diamino-6-nitrotoluene) with known retention times and UV spectra. The HPLC data of individually applied TNT compound are shown in Figure 7.11. For time- zero samples, TNT (rt = 13.5 min) and ADNTs (rt = 11–12 min) peaks were repeatedly observed in aqueous samples; but, 2,4-diamino-6-nitrotoluene, 2,4-DNT, and 2,6-DNT were not detected in any samples. Also, a peak of TNT derived catechol was consistently identified at rt = 5.7 min and several unidentified-metabolites were observed. The similar observations regarding metabolites of TNT degradation in DNT degrading culture have been reported by Tront and Hughes (13). Additionally, peaks that one more hydrophobic components than TNT were observed (rt = 18 min).

The fate of TNT in each microcosm was identified through separation and fractionation with end-point samples via HPLC (Figure 7.12). Similar observation was shown for TNT alone and TNT/2,4-DNT mixture reactors.  $^{14}\text{C}$  associated at TNT peak disappeared in time, and a small fraction of  $^{14}\text{C}$  was shown at ADNTs peaks. TNT derived catechol was consistently observed at much higher levels than ADNTs. The bulk of  $^{14}\text{C}$  was distributed in the unknown polar products after incubation of 35 days. These results demonstrate that TNT was likely metabolized by oxidative pathways through the catechol intermediate. 98.3 % and 99.5 % of  $^{14}\text{C}$  material was recovered for fractionation events in TNT alone and mixture reactors, respectively.



**Figure 7.11.** Initial HPLC–UV profiles for aqueous samples from aerobic microcosms which contained radiolabeled  $^{14}\text{C}$ -TNT, unlabeled TNT, and effluent from CSTR systems.





**Figure 7.12.** UV profile (Top) at  $\lambda = 246$  nm with corresponding  $^{14}\text{C}$  profile (Bottom) for microcosm sample.

## 7.5. Conclusions

The study presented herein examined TNT transformation during DNT mineralization through batch and CSTR studies. Unfortunately, the stoichiometry of TNT cometabolism during DNT mineralization could not be defined, but DNT degrading mixed cultures could grow on TNT under constant DNT degradation. TNT was metabolized under aerobic condition, but the transformation of TNT paralleled the production of polar metabolites, catechol, and less ADNTs. Low mineralization was also observed. This suggests oxidative transformation of TNT occurred, but 2,4-DNT cosubstrate was ineffective to acceleration of TNT mineralization. In addition, 2,4-DNT degradation was not affected by TNT, while TNT inhibited 2,6-DNT degradation. This suggests that TNT will increase recalcitrance of 2,6-DNT.

## 7.6. References

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## CHAPTER 8

### Pilot Scale Systems: 2,4,6-Trinitrotoluene Leaching and Degradation Processes

#### 8.1. Overview

Two identical pilot systems (termed TNT BioPilot 1 and 2) were operated to evaluate TNT leaching and possible degradation undergoing water infiltration using historically contaminated soils containing  $34,518 \pm 4581$  and  $23,227 \pm 2534$  mg/kg of 2,4,6-Trinitrotoluene (TNT), respectively. Each pilot system consisted of four vessels in series; first a soil tank for TNT contaminated soils, second a contact tank, third a recycle tank for water recirculation, and fourth an activated carbon tank for adsorption of nitroaromatics. There were no chemical additions (*e.g.*, nutrients) to pilot systems, and TNT, nitrite and nitrate were monitored from the outlet of each tank. Results showed the TNT leaching was less than anticipated ( $< 1$  % removal) and did not explain losses in the soil (34 and 63 % removal for System 1 and 2, respectively). The production of multiple metabolites and low nitrite/nitrate evolution were also observed at aqueous samples. This suggests that processes other than leaching contributed to TNT removal in pilot systems.

Microcosm studies using  $^{14}\text{C}$ -TNT amended effluent sample were conducted to investigate the fate of TNT metabolism. TNT transformation was observed in the active reactors and known metabolites were identified by UV spectra and retention time with standard materials. The primary metabolite was 3-methyl-4,6-dinitrocatechol with a large amount unidentified metabolites characterized as polar metabolites. Low mineralization levels ( $1.49 \pm 0.23$  %  $^{14}\text{CO}_2$ ) were observed even after complete removal of TNT.

## 8.2. Introduction

TNT remediation processes using chemical or physical treatment is costly and inefficient (1-3). Biological treatment has the potential to be more cost efficient method for disposing of TNT, thus it has been investigated by numerous researchers in several biological engineering systems over the past decades (4-6). The many published results concerning the biological fate of TNT in microbial systems agree that the aromatic ring of the TNT is not cleaved but undergoes transformation. Most commonly, reports of TNT transformation occurs through sequential reduction of nitro groups by several nonspecific nitroreductases to form nitroso- and hydroxylamino- and amino derivation (5, 7-11). After nitro-reduction, further metabolism and ring-cleavage via mono-oxygenases and dioxygenases has been characterized to a limited degree (12). Enzymes catalyzing these reactions under aerobic conditions have been reported for several nitroaromatics (13, 14). Recently, Tront and Hughes investigated a novel pathway for TNT oxidative metabolism (15). The results showed the ability of microorganisms to oxidize TNT directly through removal of a nitro group and oxygenation of the aromatic ring with one identical metabolite, 3-methyl-4,6-dinitrocatechol.

This study presents evidence for TNT-degradation at a historically contaminated site under aerobic conditions. Pilot leaching and  $^{14}\text{C}$ -tracer studies were conducted with TNT contaminated soil obtained from field site (Barksdale, WI). The leaching concentration of TNT was monitored and compared with losses in BioPilot soils at the end of this experiment. To assess the mineralization of TNT, the evolution of  $^{14}\text{CO}_2$  from microcosms containing  $^{14}\text{C}$ -TNT was monitored, and several metabolites were identified using UV spectra and retention times of standard materials. Mass balances were further

examined through separation and fractionation of  $^{14}\text{C}$ -labeled metabolites to determine the metabolic fate of the labeled TNT.

### **8.3. Materials and Methods**

#### **8.3.1. Chemicals.**

All radio-labeled materials and other chemicals were described in Chapter 7.

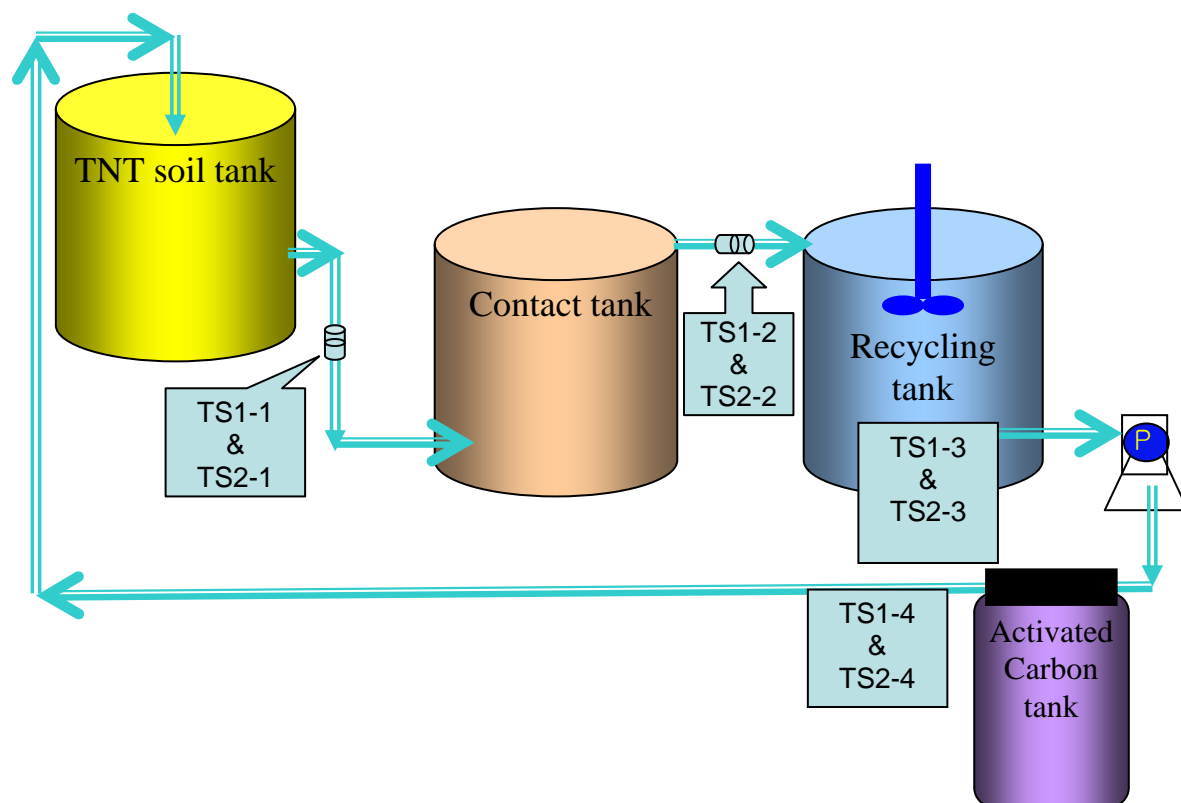
#### **8.3.2. TNT BioPilot Experimentation.**

##### **8.3.2.1. BioPilot Design.**

Figure 8.1 is a schematic of the pilot scale systems. Each system consists of four tanks. The first three vessels (a soil tank, a contact tank, and a recycle reactor) were constructed in series as described in Chapter 5, following by an activated carbon tank. TNT contaminated soils were collected from Barksdale at WI and 359 kg and 352 kg were loaded into soil tank 1 and 2, respectively. The contact tank, instead of DNR tank (Chapter 5) for nitrite/nitrate control, contained large gravel media (granite) and was operated in upflow. Water was purged from the recycle tank, through activated carbon to remove nitroaromatics in the water into the soil tank. Each system contained four sampling points: outlet from soil tank (1), outlet from contact tank (2), outlet from the recycle tank (3), and outlet from activated carbon tank (4).

##### **8.3.2.2. Experimental Protocol and Analysis.**

The feed/recycle tank was initially filled with tap water (180 Gal) and 10 Gal/day was applied to both soil tanks. Effluent discharge was hydraulically controlled to



**Figure 8.1.** Schematic diagram of TNT BioPilot (TS 1, pilot system 1; TS 2, pilot system 2) with four sampling ports.



maintain a constant water volume in the contact and recycle tank. No nutrient addition or pH control was conducted. TNT, nitrite, nitrate, and pH were monitored at each sampling point. Methods presented in previous Chapters were used for sample analysis. Nitroaromatic compounds were separated on a Zorbax® SB-C18 and nitrite/nitrate concentrations were determined by colorimetric method or Dionex IC.

Soil cores were extracted to estimate background TNT and endpoint TNT for mass balance calculations. Soil extraction procedures were described in Chapter 5.

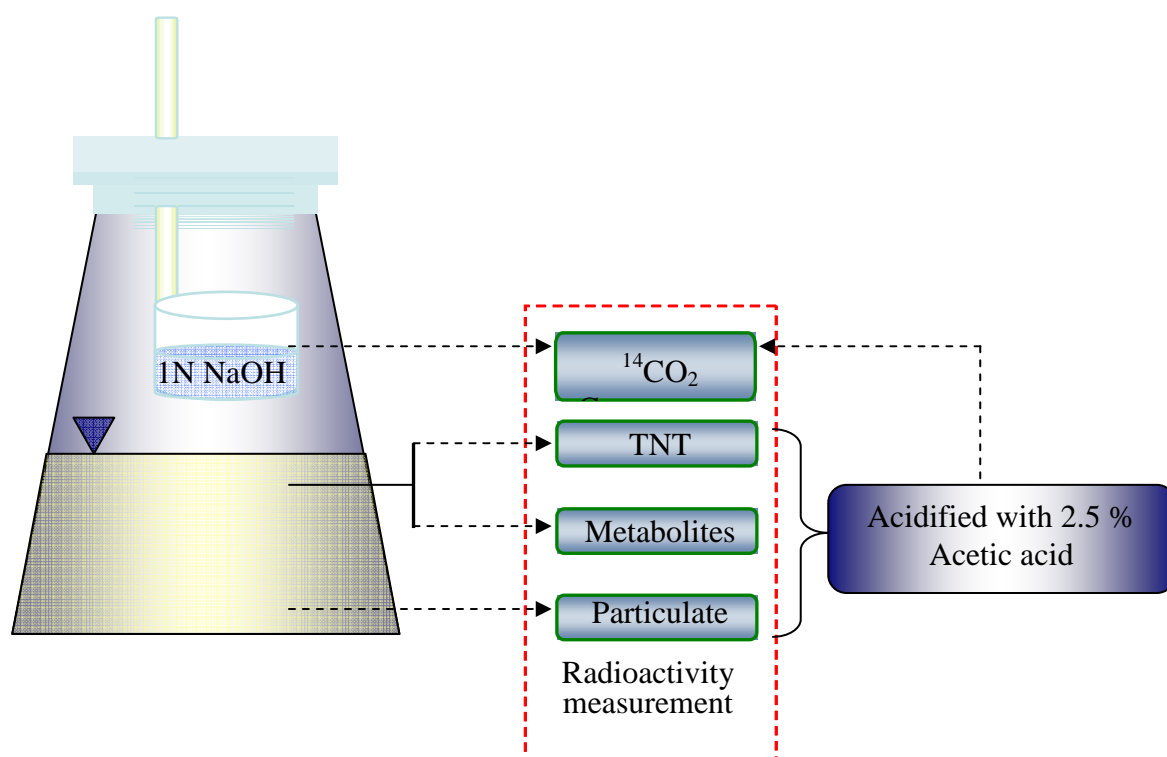
### **8.3.3. *Radioactive Tracer Experimentation.***

Experimental reactors were sealed with a PTFE lined butyl rubber septa and center well that penetrated the stopper of the reactor, which designed at the previous radioactive tracer studies (Chapter 7). For the measurement of  $^{14}\text{CO}_2$  evolution, a center well containing 1.0 N NaOH was added. The reactors were fed with unlabeled-TNT and  $^{14}\text{C}$ -TNT (18  $\mu\text{Ci/L}$ ). Two active reactors and one inactive control were run for each experimental set. Combined inhibitors (200 mg/L  $\text{NaN}_3$  and 10  $\mu\text{M}$   $\text{HgCl}_2$ ) were added in inactive control.

Microcosm samples were analyzed using the procedures presented in Chapter 7. The particulates, TNT, metabolites, and  $^{14}\text{CO}_2$  were fractionated and  $^{14}\text{C}$  was quantified by liquid scintillation counter (LSC) (Figure 8.2).

## **8.4. Results and Discussion**

### **8.4.1. *TNT BioPilot Studies***

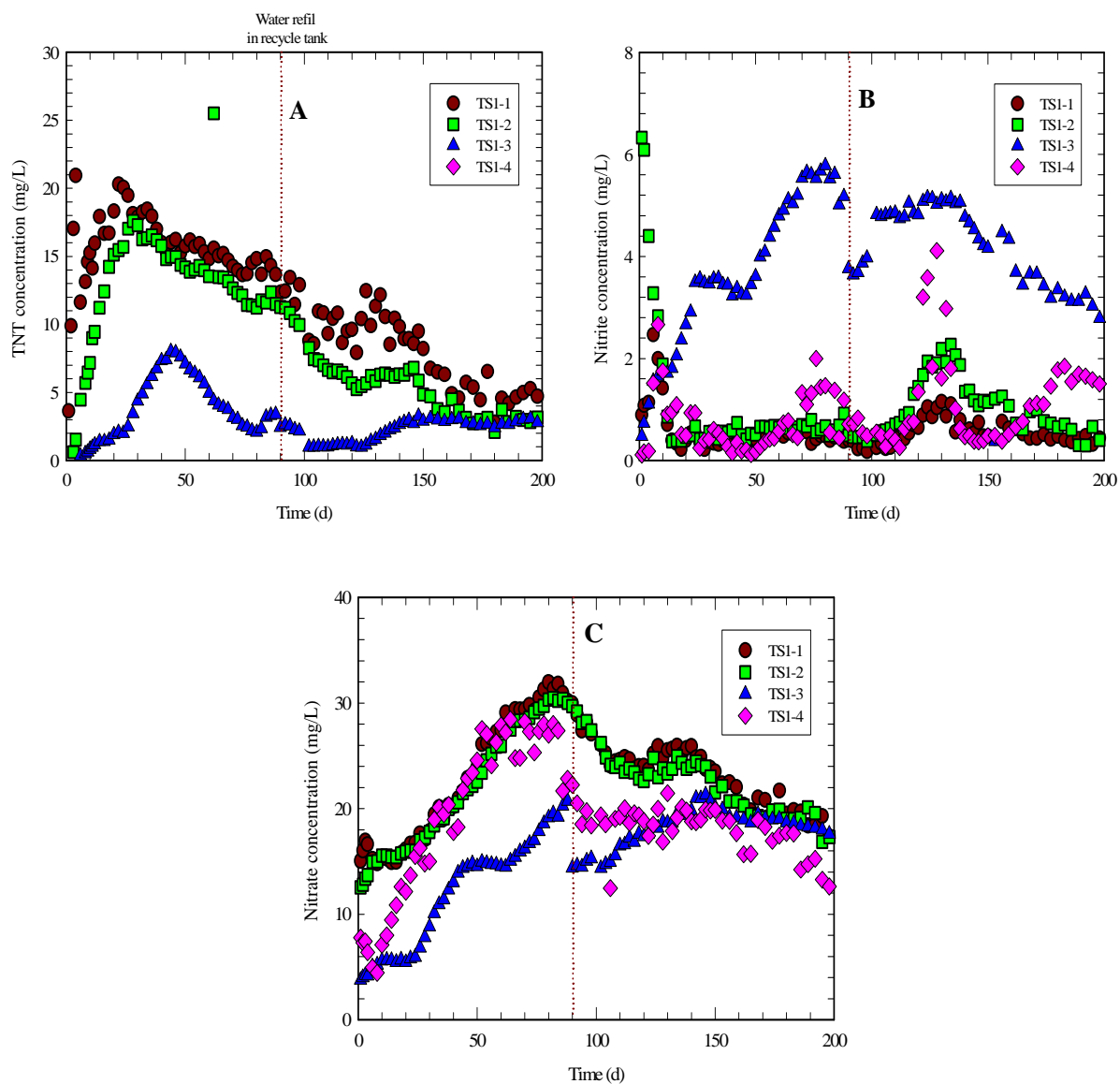


**Figure 8.2.** Procedures for the analysis of radioactive batch reactor effluents. The each radioactivity of biomass, TNT, metabolites, and  $^{14}\text{CO}_2$  were measured separately by liquid scintillation counter (LSC)

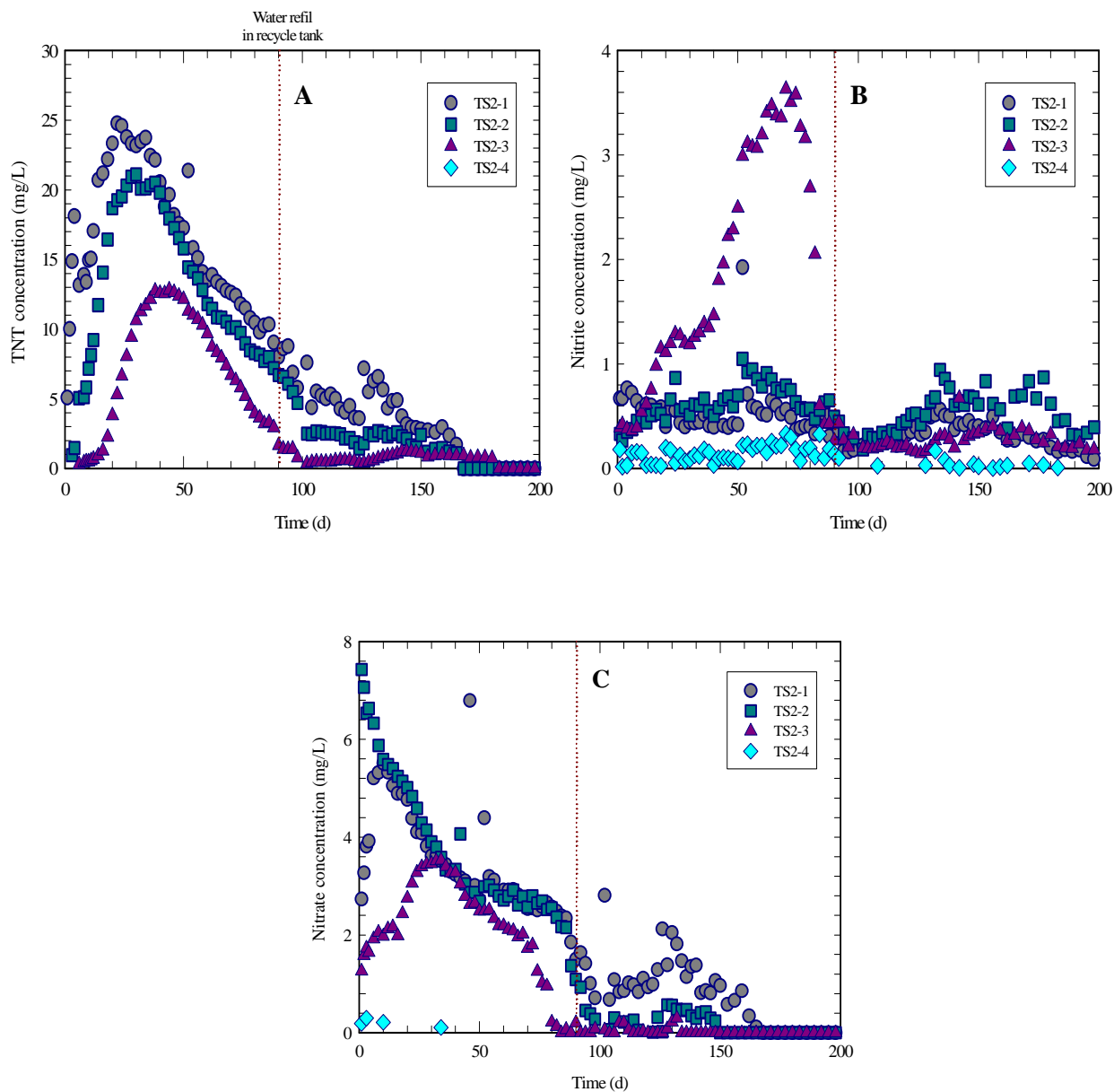
#### **8.4.1.1. Effluent Analysis.**

TNT, nitrite, and nitrate profiles are presented in Figure 8.3 and 8.4. Effluent DNT isomers (*i.e.*, 2,4-DNT and 2,6-DNT) were never detected at all sampling points over the entire 200 day operation. TNT was immediately identified at the first three sampling points (*i.e.*, outlet of soil tank, contact tank, and recycle tank) and began to increase (Figure 8.3A and 8.4A). Overall, TNT effluent concentrations increased initially, and then decreased slowly, where TNT concentrations in recycle tank were lower than that in other tanks (*i.e.*, soil tank and contact tank). On day 90, tap water was refilled in recycle tank due to reduction of water level caused by evaporation and an immediate dilution of TNT concentration was expected and observed. By the end of operation, TNT concentrations at each sampling point had stabilized between 3 and 5 mg/L for System 1 and near detection limits for System 2. No TNT was ever detected at the outlets from activated carbon tanks over the entire experiment, and pH ranged from 6.3 to 9.0 during these studies.

Nitrite concentrations from sampling point 1, 2, and 4 were often varied but typically below 2 mg/ L and 1 mg/L in System 1 and 2, respectively. In the recycle tanks, higher levels of nitrite were observed with the TNT leaching. For example, the level of nitrite in the recycle tank (TS1-3) rose to 6 mg NO<sub>2</sub><sup>-</sup>/L and stabilized at approximately 5 mg NO<sub>2</sub><sup>-</sup>/L at day 100, and then decreased slowly to 3 mg NO<sub>2</sub><sup>-</sup>/L (Figure 8.3B). Different behavior was observed at system 2 (Figure 8.4B). Nitrite levels in the recycle tank of System 2 (TS2-3) increased initially, but dropped suddenly below 1 mg/L at day 84. Low levels of nitrite were observed at outlet from other tanks over the entire experiment.



**Figure 8.3.** The profiles of effluents in TNT BioPilot System 1: (A) TNT effluent concentrations (B)  $\text{NO}_2^-$  effluent concentrations (C)  $\text{NO}_3^-$  effluent concentrations. 1, 2, 3 and 4 represent sequential tank sampling ports for each system.



**Figure 8.4.** The profiles of effluents in TNT BioPilot System 2: (A) TNT effluent concentrations (B)  $\text{NO}_2^-$  effluent concentrations (C)  $\text{NO}_3^-$  effluent concentrations. 1, 2, 3 and 4 represent sequential tank sampling ports for each system.

The levels of nitrate were monitored during the studies because nitrifying bacteria were likely present in the field soil as demonstrated at previous studies (Chapter 3 and 4). Nitrate was gradually produced and concentrations stabilized for all sampling points at System 1 (Figure 8.3C). Concentrations of nitrate ranged from 3.78 to 31.97 mg/L and eventually equilibrated after water-refill. Different results were observed in System 2 (Figure 8.4C). The level of nitrate decreased steadily after initial elevation, and then was sustained at low concentrations. Over the entire operation, nitrate ranged from below detection limits to 7.43 mg/L in System 2.

#### **8.4.1.2. Endpoint Assessments: Soil Core and Final Discharge Analyses.**

Table 8.1 presents background TNT concentrations prior to operation and remaining concentrations in soils at the completion of the pilot studies. Considerable TNT was removed to soil in both systems after treatment during 200 days. The percent of removal for Systems 1 and 2 was 63 % and 34 %, respectively, by comparison of soil samples from time = 0.

Using effluent concentrations from soil tank effluents, at a leaching rate of 10 Gal/d, the mass of TNT that leached from the tank was estimated. From leaching, TNT mass removal over the entire operation was 85,376 mg TNT in Soil Tank 1 and 68,985 mg of TNT in Soil Tank 2, representing a loss of < 1 % for both systems. The estimation of removal differed significantly to results of soil extraction and leaching did not explain TNT losses from the soil. This suggests that TNT losses in the pilot systems are attributed to mechanisms other than leaching.

**Table 8.1.** Soil extraction results of TNT and DNTs: background concentrations prior to operation vs. remaining TNT concentrations after 200 d treatment.

<b>I.</b>	Sample Name	TS1	TS2
	DNTs	Trace	Trace
	mg/Kg TNT in dry soil (Average $\pm$ Std Dev.)	34,518 $\pm$ 4581	23,227 $\pm$ 2534
<b>II.</b>	Sample Name	TS1	TS2
	DNTs	ND	ND
	mg/kg TNT in dry soil (Average $\pm$ Std Dev.)	12,789 $\pm$ 1622	15,343 $\pm$ 620
	% of Removal	63	34

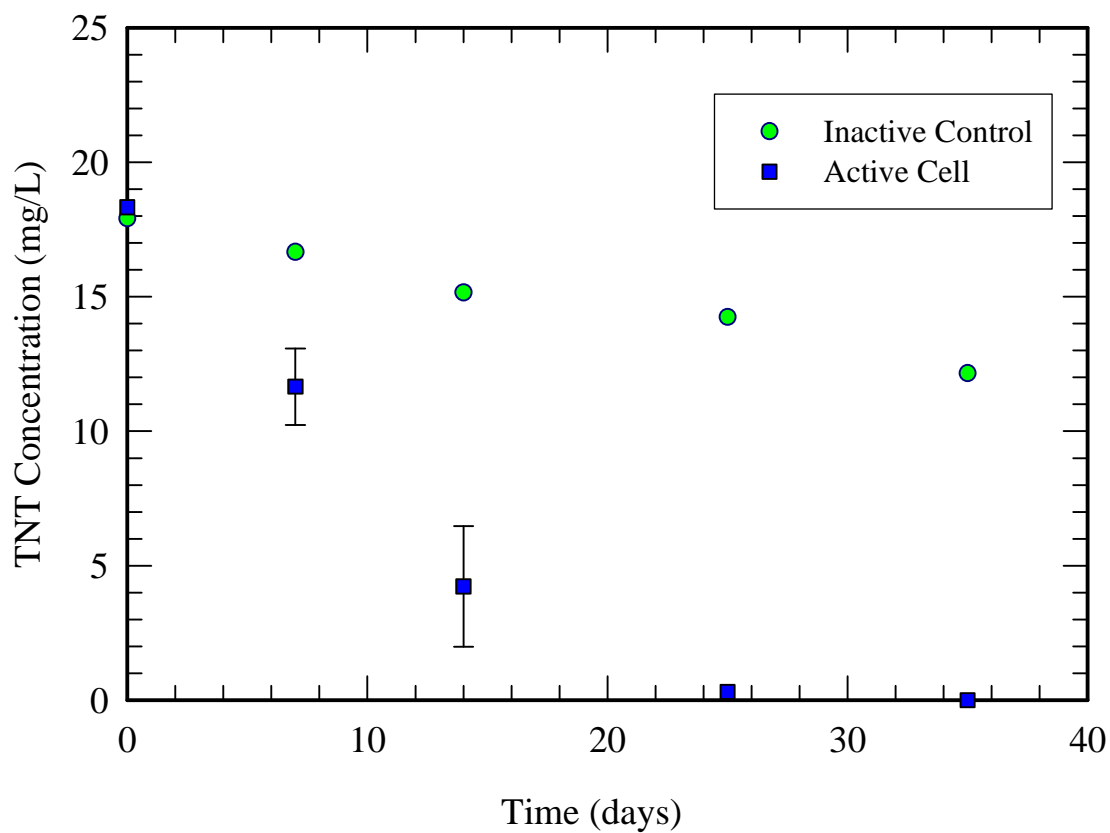
I, Background TNT and DNT in bulk soil (*i.e.*, Initial TNT/DNT concentration with dry soil); II, TNT and DNT concentrations in soil treated for 200 d.

**8.4.2.  $^{14}\text{C}$  Tracer Studies: Degradation of TNT in Microcosms.** To demonstrate TNT losses in the pilot systems, microcosm studies were employed with effluent samples collected from the recycle tank (TS1-3). TNT was the sole carbon and nitrogen source. Active microcosms demonstrated complete removal of both unlabeled TNT and  $^{14}\text{C}$ -TNT under aerobic conditions (Figure 8.5). TNT degradation followed first-order kinetics and > 98 % of TNT was transformed in active microcosms within 25 d. Controls with sodium-azide exhibit less loss of TNT. To extend the hypothesis that indigenous microorganisms from Barksdale site may mineralize TNT aerobically, the production of  $^{14}\text{CO}_2$ ,  $^{14}\text{C}$  incorporated with particulate, and accumulation of aerobic metabolite (*i.e.*, 3-methyl-4,6-dinitrocatechol) were evaluated in microcosms. The cumulative  $^{14}\text{CO}_2$  profile at each successive time point showed no appreciable mineralization over the overall study (Figure 8.6). The maximum rate of mineralization was estimated at 0.29 %  $^{14}\text{CO}_2/\text{d}$  (Logarithm,  $R^2 = 0.98$ ).

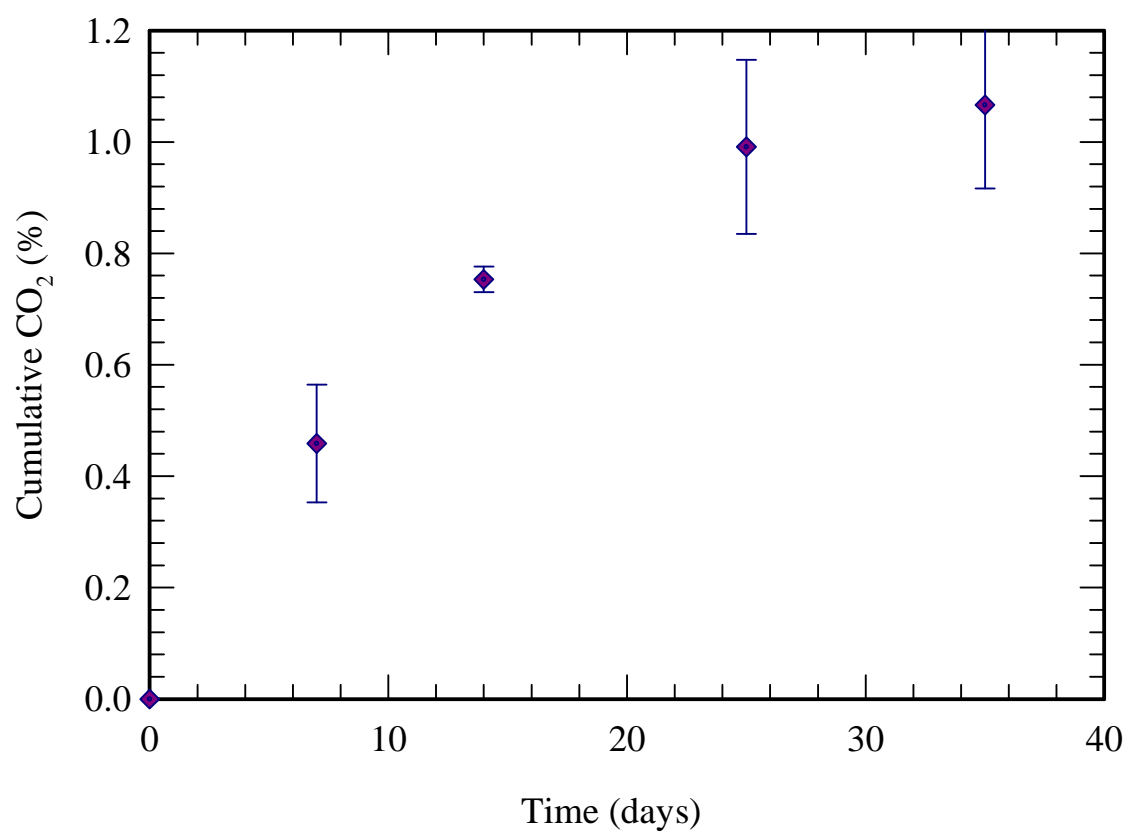
It is important to note that pH in the reactor was buffered to 7.5–8.0; therefore, large fraction of inorganic carbon (*i.e.*,  $\text{CO}_2$ ) was expected in the aqueous phase. To further demonstrate the quantifiable mineralization of TNT in reactors, reactors were acidified and  $^{14}\text{CO}_2$  was counted at the end of experiment (35d). Data for  $^{14}\text{C}$  distribution are presented in Table 8.2. Low level of mineralization ( $1.5 \pm 0.2$  %  $^{14}\text{CO}_2$ ) was observed in active microcosms within 35 d and TNT-derived carbon was minimally incorporated into particulate ( $3.4 \pm 3.0$  %). Most of  $^{14}\text{C}$  in active reactors was present as aqueous phase ( $79 \pm 7.3$  %).

Separation and fractionation of  $^{14}\text{C}$ -labeled metabolites via HPLC were conducted for examination of product distribution (Figure 8.7). 3-methyl-4,6-dinitrocatechol (5.7





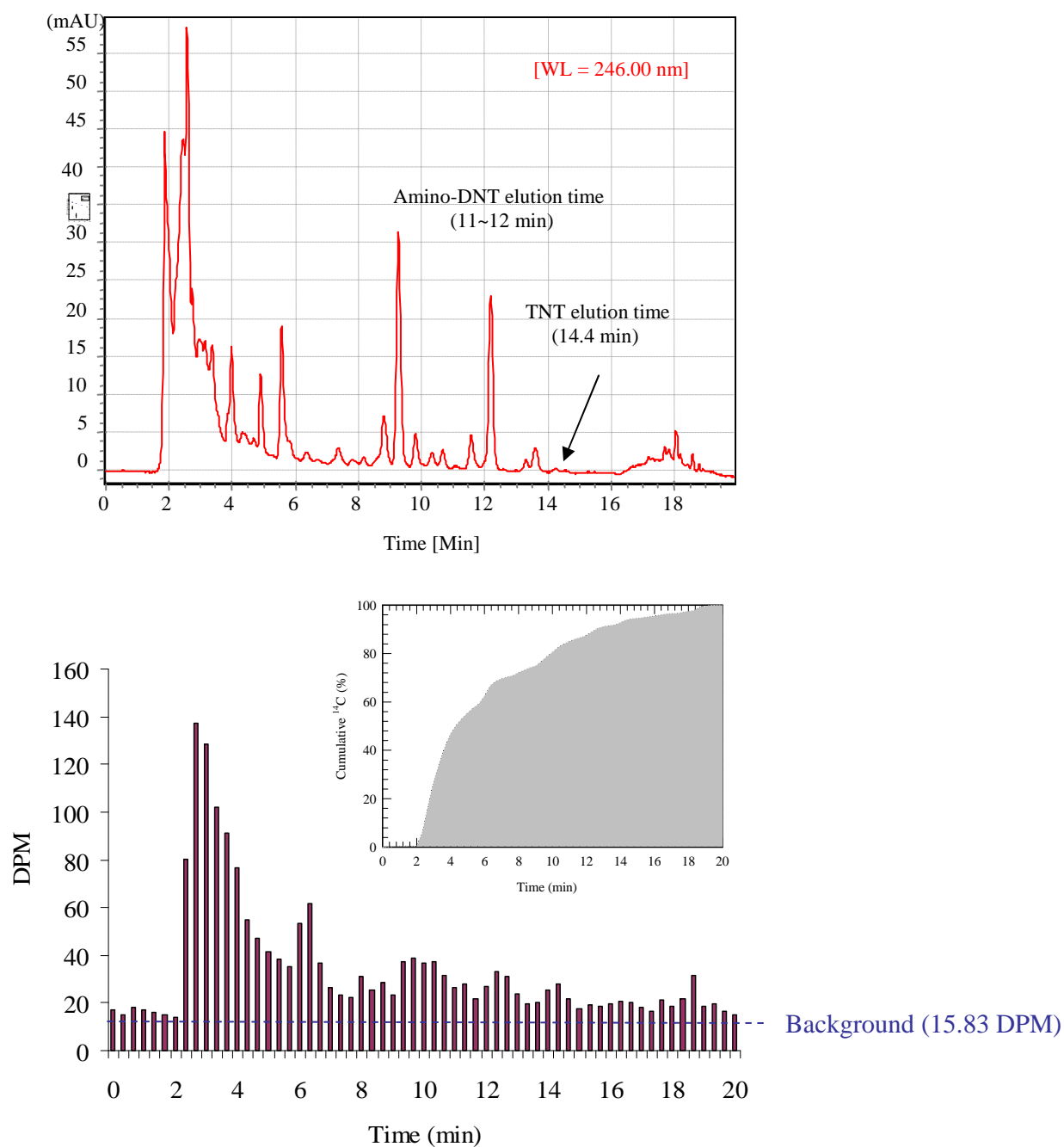
**Figure 8.5.** Batch studies: TNT degradation shown by inoculum obtained from TNT BioPilot System (TS 1-3). TNT was used as sole carbon and nitrogen source.



**Figure 8.6.** Percentage mineralization of  $^{14}\text{C}$ -TNT to  $^{14}\text{CO}_2$  in microcosms containing inoculum from “Barksdale” TNT BioPilot before acidification.

**Table 8.2.** Distribution profiles of  $^{14}\text{C}$  radioactivity in microcosms with TNT alone after 35 d.

Radioactivity (%) – Microcosms containing TNT alone				
	Total $^{14}\text{C}$	$^{14}\text{CO}_2$	Aqueous $^{14}\text{C}$	$^{14}\text{C}$ -particulate
Active Reactor	$89 \pm 5.9$	$1.5 \pm 0.2$	$79 \pm 7.3$	$3.4 \pm 3.0$
Killed Control	93	0.7	94	2.7



**Figure 8.7.** HPLC-UV profile (Top) at  $\lambda = 246$  nm with corresponding  $^{14}\text{C}$  fractionation results (Bottom) for an active microbial system; distribution of  $^{14}\text{C}$  from aqueous sample in reactor incubated for 35 d under aerobic conditions.

min) and amindinitrotoluenes (ADNTs, 11 – 12 min) consistently eluted. TNT peak (14.4 min) decreased and completely depleted, and the polar compounds were accumulated. The compounds diaminonitrotoluene, 2,4-DNT, and 2,6-DNT were not detected. The  $^{14}\text{C}$  was mostly associated with the polar front (59 %) and small portion of  $^{14}\text{C}$  (4 %) was associated with the ADNTs in spite of apparent peaks in UV profiles. TNT and 3-methyl-4,6-dinitro catechol were observed at 2 % and 8 % of  $^{14}\text{C}$ , respectively. Overall, 95.2 % of  $^{14}\text{C}$  was recovered. Through radioactive tracer results associated with identification of oxidative metabolite (*i.e.*, 3-methyl-4,6-dinitro catechol) and mostly polar compounds. Similar observations regarding oxidative microbial degradation of TNT has been reported by Tront and Hughes (15).

The small amounts of  $^{14}\text{C}$  in particulate material are interesting.  $^{14}\text{C}$ -particulate does not represent a significant yield-value for biomass growth. This fraction may be due to humic substances originating from field soils (16, 17). A significant number of HPLC peaks appeared later than the TNT peak (14.4 min), and are more hydrophobic than TNT. Some of  $^{14}\text{C}$  was also distributed at this region. The possible humus incorporation of  $^{14}\text{C}$ -TNT may occur due to some direct incorporation of catechol intermediates with natural organic matter (NOM), as catechols for humus incorporation can be stimulated by methyl substituents (16). In addition, the high molecular weight hydrophobic compounds may involve polymeric material. If the aromatic ring is not cleaved, and the nitro groups are reduced, hydroxylamino-intermediates can couple to form azoxy compounds containing  $^{14}\text{C}$  derived from TNT (2, 5, 18). These studies have not offered direct evidence to verify of these possible mechanisms, so all must be considered. Considerable research of aerobic TNT metabolism will be required before specific loss mechanism can be verified.

In summary, TNT loss from leaching was observed with effluent concentrations well below saturation in the two identical pilot systems. Correspondingly, nitrite and nitrate was observed at low or appreciable levels in both systems, which do not explain TNT losses through oxidative biodegradation. Leaching calculation and soil extraction demonstrated that TNT was minimally removed through leaching, while significant TNT was removed in soils.  $^{14}\text{C}$  experimentation presented the loss of  $^{14}\text{C}$ -TNT, the production of 3-methyl-4,6-dinitrocatechol,  $^{14}\text{C}$  increasingly associated with polar front in HPLC sample, and other unknown metabolic products with distinct and small peaks. Despite complete depletion of TNT, low mineralization was observed as measurement of low  $^{14}\text{CO}_2$ .

## 8.5. Conclusions

The leaching concentration of TNT was far lower than anticipated based on TNT solubility limits and the high concentrations in the soil. Additionally, the net loss of TNT did not account for an appreciable percentage of the TNT losses in the soil that were observed. This latter observation suggests that an additional process(es) contributed to TNT removal in the soil reactors in both pilot systems. To further understand the mechanism(s) of TNT loss,  $^{14}\text{C}$  tracer studies were conducted in microcosms established with pilot system effluent samples. Results demonstrated that microbes indigenous to the system transformed TNT through known and unknown intermediates with low mineralization extent ( $1.5 \pm 0.2 \%$ ). Possible mechanisms for TNT disappearance were evaluated, based on literature reports (such as composting processes, anaerobic TNT

degradation, and aerobic TNT degradation) in an attempt to explain the high levels of TNT lost in the soil bioreactor. These are discussed in the following paragraphs.

The loss of TNT in the soil system, not accounted for by leaching is similar to results of composting systems (19-22). To initiate composting processes, TNT contaminated soil is mixed with degradable organic material (*e.g.*, leaves, straw, wood chips, hay, etc.), and the appropriate amount of moisture is maintained (23). Composting processes yield a transformation of TNT to various amino-reduction products and the formation of azoxy-compounds. The net effect is the disappearance of TNT is through the binding/sorption of transformation products to soil components, and the formation of unbound residues (22, 24). However, in the pilot system, there was no addition of degradable organic material into TNT biopilots, existing COD appeared to be highly refractory, and TNT derived  $^{14}\text{C}$  in microcosms was dominated by highly polar, soluble compounds and not the compounds considered as those primarily produced in composting. For these reasons, it is not possible to describe the loss of TNT through processes analogous to those occurring in composting.

A second possible mechanism for TNT disappearance is anaerobic transformation in soils. The intermediates most commonly formed during anaerobic TNT biotransformation processes include ADNTs and DANTs, and formation of TAT has been observed under strong reducing conditions (even when TAT is not detected, it is quickly postulated as a central intermediate in the anaerobic treatment of TNT) (11, 25). In this study, DANTs were never observed, but ADNTs were repeatedly identified at low levels through comparison of UV spectra and retention time with standard samples. ADNTs concentrations did not vary over time (*i.e.*, they did not accumulate) and their

concentration in leachate can not account for the levels of TNT loss observed. The observation of ADNTs, with out DANTS is consistent with reduction processes that can occur with TNT under aerobic conditions. It is well known that the reduction of one nitro-group on TNT is possible under aerobic conditions (15, 22). In aerobic microcosms, ADNT production was found to be a dead-end product, that accounted for only a small fraction of the TNT transformed, and it appears that the ADNT produced was only a minor fraction of the net TNT removal and is consistent with the activity of reductase activity in aerobic bacteria.

TNT loss mechanisms through aerobic fungal biodegradation have been shown, which begins with hydroxylation followed by ring cleavage and finally mineralization. Certain fungi fortuitously can degrade TNT through lignolytic degradation of TNT and fungi-mediated radical attack on the aromatic ring after reduction of an aryl nitro group (15, 22). These two mechanisms are among the few known that lead to the mineralization of TNT (15, 22). However, high concentrations of TNT in the contaminated media and the lack of appropriate added substrates (*e.g.*, corn cobs, etc) limits the activity of a lignolytic community (26). Herein, there were no substrates added to the soil to enrich TNT degrading fungi using ligninolytic growth substrates. It is reported that fungi growing under ligninolytic conditions can mineralize TNT up to 30 % levels (22), while much lower mineralization was observed in this study. As a result, fungal degradation of TNT did not likely play a significant role in TNT disappearance observed in soils.

Observations made by Tront and Hughes (15) are strikingly similar to observed mechanisms for TNT loss, and strongly support oxidative degradation of TNT in this



pilot study. In their work, they demonstrated that a mixed 2,4-DNT degrading cultures oxidatively metabolized TNT through a corresponding catechol with release of the nitro group as nitrite. Low mineralization was observed as measured by CO<sub>2</sub>. Shake flask experiments constructed with soil from historically contaminated sites (*i.e.*, Barksdale, WI) also showed parallel disappearance of TNT and production of TNT derived catechol and nitrite. These observations indicated that 2,4-DNT degrading mixed cultures may carry out oxidative attack of TNT and that growth on TNT is plausible. Based on literature available, this loss mechanism appears most likely to be the factor that contributed to TNT loss in the biopilot system.

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## CHAPTER 9

### CONCLUSIONS

The goal of this research was to improve the understanding of the limits and effectiveness of *in situ* bioremediation and natural attenuation (NA) of DNT, and to examine the metabolism of TNT in the presence of DNT mixtures, and TNT leaching characteristics under *in situ* treatment condition. Specific conclusions are presented in the following lists divided by the chapter in which experiments derived the conclusion.

#### **9.1. *In Situ* Bioremediation of DNT Contamination in Historically Contaminated Soils**

- Indigenous cultures capable of mineralizing DNT were present in Barksdale soils.
- 2,4-DNT mineralization was initiated and sustained in soil columns. However, limited 2,6-DNT mineralization was observed due to the presence of 2,4-DNT.
- Variation of evolved N-ratio (*i.e.*,  $\text{NO}_3^-/\text{NO}_2^-$  ratio) was observed and was controlled by DNT levels in the soils.
- Factors controlling mineralization in column and batch studies included:
  - pH (at and below 6.0) reduced degradation activity.
  - Elevated nitrite concentrations ( $\geq 5$  mM) inhibited degradation activity.
  - Enhanced buffer strength (capacity) increased and maintained biodegradation.
  - Mineral media addition did not significantly stimulate degradation activity.

- Enhancing aeration produced slightly increased degradation activity (when 2,4-DNT was present).

## **9.2. Monitoring Performance and Factors Controlling DNT Degradation in Vadose-Zone Simulations**

- The immediate biodegradation of both DNT isomers was observed in initial bioaugmented column studies where fed DNT concentrations near saturation under experimental conditions (oxygen and other nutrient requirements are available).
- Factors controlling mineralization in vadose zone through column and batch studies included:
  - pH, at 5.5, strongly inhibited DNT degradation.
  - Nitrite concentration of 60 mM and above severely limited the biodegradation of 2,4-DNT. However, 2,6-DNT degraders were less sensitive.
  - Longer HRT enhanced the extent of mineralization.
  - Aeration capacity had a minimal impact on biodegradation of DNT within columns operated with field capacity.
- While the pH effect could be reversed for 2,4-DNT degrading systems by raising the influent pH, the 2,6-DNT recovery was slow and inconsistent indicating that pH inhibition may have extended effects on 2,6-DNT degradation processes.
- Nitrite evolved from columns was detected but at less than stoichiometric amounts due to biological nitrification processes converting nitrite to nitrate.

### 9.3. DNT BioPilot Studies: DNT Bioremediation Coupled with Denitrification Processes

- 2,4-DNT and 2,6-DNT were readily biodegraded in this soil pilot system under conditions that simulate water infiltration. Over 95 % of both DNT isomers were removed after 100 d.
- Treatment of nitrogen evolution associated with DNT degradation was achieved through the addition of degradable COD.
- An increase of 2,6-DNT concentration in the pilot effluent was observed due to recycled COD.
- Soil effluent contained recalcitrant nitroaromatics (*i.e.*, Dinitroxyline isomers as identified by GC-MS) and unidentifiable organics (possibly a reduced form of DNT) which remained present even in extended microcosm studies.

### 9.4. Natural Attenuation of DNTs: Determination of Biodegradation Limits

- Biodegradation limits of 2,4-DNT and 2,6-DNT were obtained experimentally. The limiting 2,4-DNT concentrations for chemostat and column system were  $0.052 \pm 0.002 \mu\text{M}$  and  $0.060 \pm 0.004 \mu\text{M}$ , respectively. For 2,6-DNT, the limiting value for chemostat studies was  $0.035 \pm 0.004 \mu\text{M}$ , and in columns the limiting value achievable was  $0.026 \pm 0.004 \mu\text{M}$ .
- Similar limiting values for each DNT isomer were estimated from chemostat and column system, which provide strong evidence that at concentrations less than biodegradation limits obtained, DNT can not support a sustained population of DNT degraders.

- Estimated limits of 2,6-DNT biodegradation was lower than those of 2,4-DNT biodegradation.
- Estimated limiting concentrations were lower than regulatory requirements recommended by the US EPA and the CFR. Degradation will occur to trace levels under appropriate conditions, where oxygen and other nutrient requirements are present.

### **9.5. Cometabolic Biotransformation of Trinitrotoluene Supported by Dinitrotoluenes under Aerobic Conditions**

- TNT cometabolism was initiated in the presence of DNT; however, high concentrations of TNT caused inhibition of TNT cometabolism within both DNT degrading cultures.
- The rate of 2,4-DNT degradation was not significantly influenced by the presence of TNT.
- The presence of TNT caused the inhibition of 2,6-DNT degradation, but low concentrations of TNT fed to 2,6-DNT degrading culture was cometabolized during 2,6-DNT degradation.
- Although TNT was completely transformed at batch reactors containing 2,4-DNT, 2,4-DNT addition did not promote mineralization of TNT ( $2.30 \pm 0.18 \%$ ).
- TNT was partially oxidized forming 3-methyl-4,6-dinitro catechol and other unidentified metabolic intermediates. Polar compounds were formed along with a small portion of the “less-polar” compounds.
- Amino-dinitrotoluenes (ADNT) were produced but only at trace level.



## **9.6. TNT Biopilot Studies: 2,4,6-Trinitrotoluene Leaching and Degradation Processes**

- TNT loss from leaching was observed with effluent concentrations well below saturation in the two identical pilot systems
- Nitrite and nitrate was observed at low or appreciable levels in both systems, which do not explain TNT losses through oxidative biodegradation.
- The removal of TNT level particularly in recycle tanks occurred along with higher evolution of nitrite and nitrate.
- Leaching calculation and soil extraction demonstrated that TNT was minimally removed through leaching, while significant TNT was removed in soils.
- $^{14}\text{C}$  experimentation presented the loss of  $^{14}\text{C}$ -TNT, the production of 3-methyl-4,6-dinitrocatechol,  $^{14}\text{C}$  increasingly associated with polar front in HPLC sample, and other unknown metabolic products with distinct and small peaks
- Products included nitrite reduced form (i.e., aminodinitrotoluenes), but low  $^{14}\text{C}$  was associated in fractionation.
- Mineralization was low ( $1.49 \pm 0.23 \%$ ) after  $> 90\%$  TNT removal (35 d).

## **9.7. Summary**

In summary, microorganisms indigenous to the Barksdale site were capable of mineralizing 2,4-DNT and 2,6-DNT. 2,4-DNT degradation readily occurred in a bench-scale system during water infiltration, but limited 2,6-DNT mineralization was observed

in the presence of 2,4-DNT. These studies explored pertinent variables with goals of quantifying and optimizing DNT mineralization activity. Low pH has a significant effect on the reduction of bioactivity, but the nitrite toxicity to DNT degraders may be reduced at field sites because of conversion of nitrite to nitrate by nitrite oxidizer. Pilot studies demonstrated that 2,4-DNT and 2,6-DNT were readily biodegraded under conditions that simulate water infiltration and denitrifying bacteria were active in the presence of biodegradable COD. Careful determination of suitable COD/N ratio was required for practical application to ensure both DNT and nitrate/nitrite removal if recycle is applied in field systems. These findings support assessment of *in situ* bioremediation to control both DNT and nitrate/nitrite. As applied at DNT plumes under appropriate conditions (oxygen and nutrient available), lower levels than regulatory limits can be reached by DNT biodegradation processes under continuous flow, non-steady state conditions.

As presented in TNT BioPilot studies, the leaching concentration of TNT was far lower than anticipated and did not explain losses in the soil. This suggests that additional processes other than leaching contributed to TNT removal in pilot systems. <sup>14</sup>C tracer studies indicate that TNT was transformed through oxidative attack, but that the extent of mineralization was very low. As a result, both leaching and oxidative metabolic processes caused removal of TNT in historically contaminated soils. Additionally, TNT cometabolism during 2,4-DNT degradation was investigated in microcosms, which indicated that 2,4-DNT cosubstrate was ineffective to acceleration of TNT mineralization. These (co-)metabolic processes can incorporate to the assessment of TNT fate at contaminated sites.

## CHAPTER 10

### ENGINEERING SIGNIFICANCE

This work provides information useful for practitioners considering *in situ* bioremediation, or NA at field scale. Soil extraction results presented herein indicated that variation caused by heterogeneity (*i.e.*, distribution of nitroaromatics or agglomerations of non-bioavailable crystalline nitroaromatics) was very high, making direct soil measurements unreliable indicators of contaminant destruction. The key factor affecting *in situ* biodegradation of DNT was system pH (*i.e.*, pH 6 or below). In biopilot studies coupling DNT degradation and nitrogen control, augmentation of degradable organics was required for treatment of  $\text{NO}_2^-/\text{NO}_3^-$  evolution associated with DNT degradation, but caused a decrease of 2,6-DNT bioactivity in recycle mode. Additionally, nitrite measurement should not be taken as a conclusive indicator of DNT degradation. Nitrite oxidizers converted nitrite into nitrate, and the N-ratio (*i.e.*,  $\text{NO}_3^-/\text{NO}_2^-$ ) was controlled by DNT levels in the soils.

The findings presented herein suggested importance of mixing, pH control, and the ratio of biodegradable COD to nitrogen (N) for *in situ* DNT bioremediation and NA. Homogeneous soil preparation through mixing is needed to improve the assessment of degradation of DNTs in soils. Mixing also inhibits compression of the soil and creates aerobic condition (*i.e.*, enhanced oxygen availability). Additionally, it can cause the distribution of buffer amendments within the soils, which help to control pH. COD/N ratio could help understanding the degree of DNT and  $\text{NO}_2^-/\text{NO}_3^-$  removal. Careful determination of suitable COD/N ratio is required for *in situ* applications. Assessment of

nitrogen balance also suggests that variation of N-ratio can be useful tool to predict *in situ* conditions of DNT availability at contaminated sites.

In TNT contaminated soils, TNT was not mineralized by indigenous microorganisms despite oxidative biotransformation. Mixed cultures capable of growth on DNT did not mineralize TNT during DNT degradation, either. This suggests that the mixtures of contamination did not improve the potential for *in situ* TNT bioremediation. Also, TNT disappearance was observed in biopilot systems containing historically TNT contaminated soils. Organisms capable of oxidative metabolism of TNT are likely distributed in the environment because TNT-derived catechol was evolved as the evidence of oxidative metabolism of TNT. However, mineralization was very low. These findings allows for predicting an important loss mechanism for TNT in *in situ* bioremediation. As a result, current thought on *in situ* bioremediation of TNT may require significant additional study.

## CHAPTER 11

### FUTURE RESEARCH RECOMMENDATIONS

This work has contributed to the understanding of potential of *in situ* bioremediation or NA of nitroaromatics (*i.e.*, 2,4-DNT, 2,6-DNT, and TNT), but there are still areas that warrant further investigation. Nitrite balances were not accurate in many cases, especially under low growth conditions where nitrite could not use as indicator for complete biodegradation. Therefore, another means for quantification of DNT biodegradation processes are necessarily required in order to propose and implement *in-situ* bioremediation and NA remedies. For example, stable isotope fractionation analysis may be used to assess the degree of degradation processes at DNT contaminated fields. Through the quantitative analysis, it can be possible to achieve accurate mass balances on DNT biodegradation in a heterogeneous soil system and identification of a predominant degradation process at a field site will be a key to understand *in situ* bioremediation and NA potential.

Another area that warrants further research is to determine the effect of various co-substrates on the TNT biotransformation/mineralization extent and rates under aerobic condition. The work conducted within this thesis demonstrated the effect of the only DNT (*i.e.*, 2,4-DNT and 2,6-DNT) cosubstrates on the TNT degradation in historically DNT degrading culture. Follow-up mixture studies are recommended with various aromatic or non-aromatic cosubstrates, which may be synergistic or antagonistic on TNT cometabolism. Identification of metabolites is required to examine their effect on breakdown/promotion of TNT degradation. The development of this cometabolic studies

will allow for the interpretation of TNT recalcitrance in field sites and hopefully improvement of TNT mineralization.

TNT (co-)metabolisms under aerobic condition were investigated using batch and pilot studies. TNT was partially oxidized via TNT-derived catechol, but many unknown metabolites were identified, which have the difficulties and limitations to confirm TNT oxidation. The oxidative metabolism of TNT poorly understood and uncharacterized can be demonstrated by the identification of the metabolites from carbon and nitrogen stable isotope analysis using GC/MS or LC/MS. Then, we can propose some possible solutions to discuss findings of TNT oxidative (co-)metabolisms in mixture and TNT BioPilot studies.

## **APPENDIX A**

The information provided herein briefly documents the history and problems of explosive contamination in Barksdale, WI based on the current conditions report for the former DuPont Barksdale work submitted in May 31 of 2007. DuPont operated the former Barksdale works facility from 1904 to 1971. The facility primarily produced dynamite, nitroglycerin and TNT for the US Military and the mining industry. Other products associated with the explosives industry were produced at the Barksdale Works in smaller quantities including Trinitroxyethylene, Trivelene, Lydol, Nitramon, soda amatol, and Nitramex. Intermediate and supplemental materials produced included sulfuric acid, nitric acid and sellite.

Production of explosives officially ceased at Barksdale in 1971. Upon termination of this, DuPont performed dismantling, demolition and clean-up activities during construction seasons through 1983. Following shutdown, the grounds became overgrown by prairie and forest vegetation. Smaller scale demolition activities occurred as late as 1985. However, investigation activities were again initiated in 1997 because a groundwater sample collected from an adjacent residential drinking water well indicated the presence of 2,4-dinitrotoluene and 2,6-DNT. In response, DuPont worked to identify affected private wells, and where warranted, installed and maintained carbon treatment systems at affected homes, so that residential drinking water met appropriate Wisconsin regulatory criteria. At the same time DuPont undertook several hydro-geological and engineering feasibility studies to determine the most appropriate long-term solution to address the affected drinking water supplies in the vicinity of the former Barksdale Works.

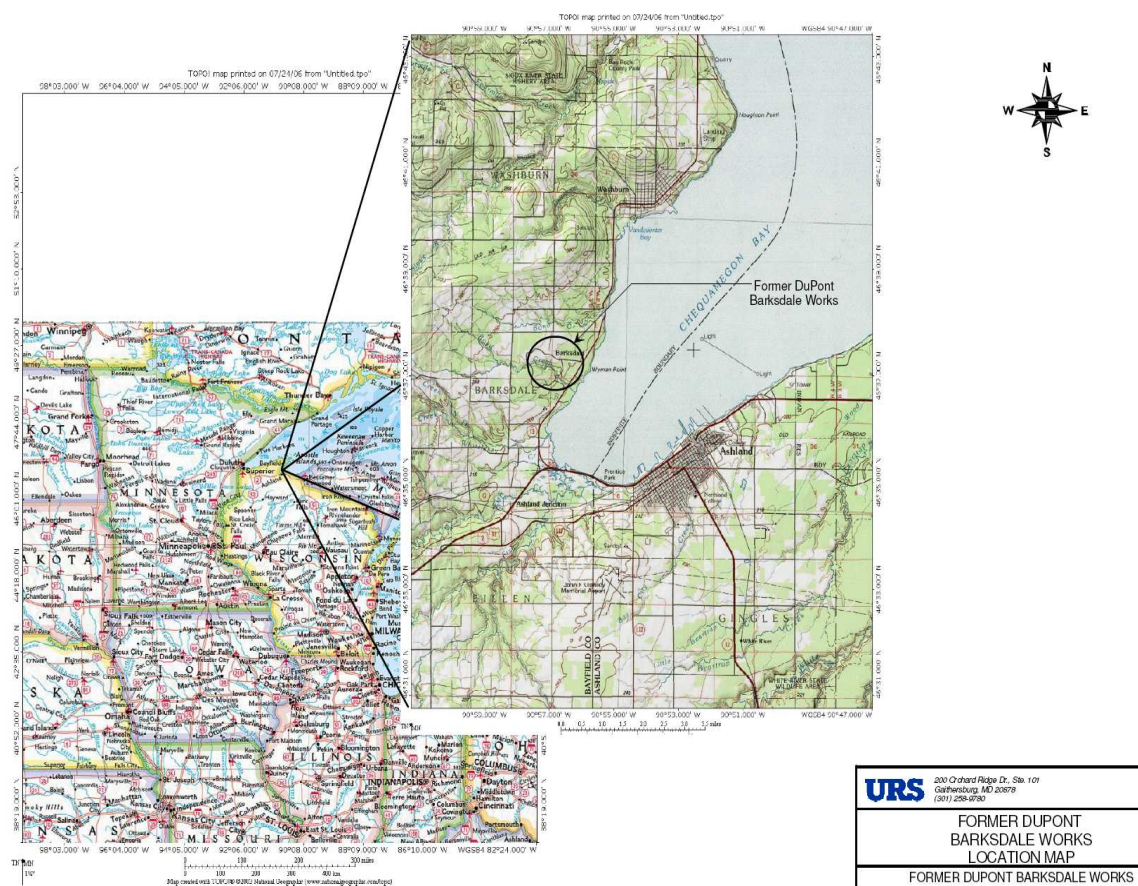
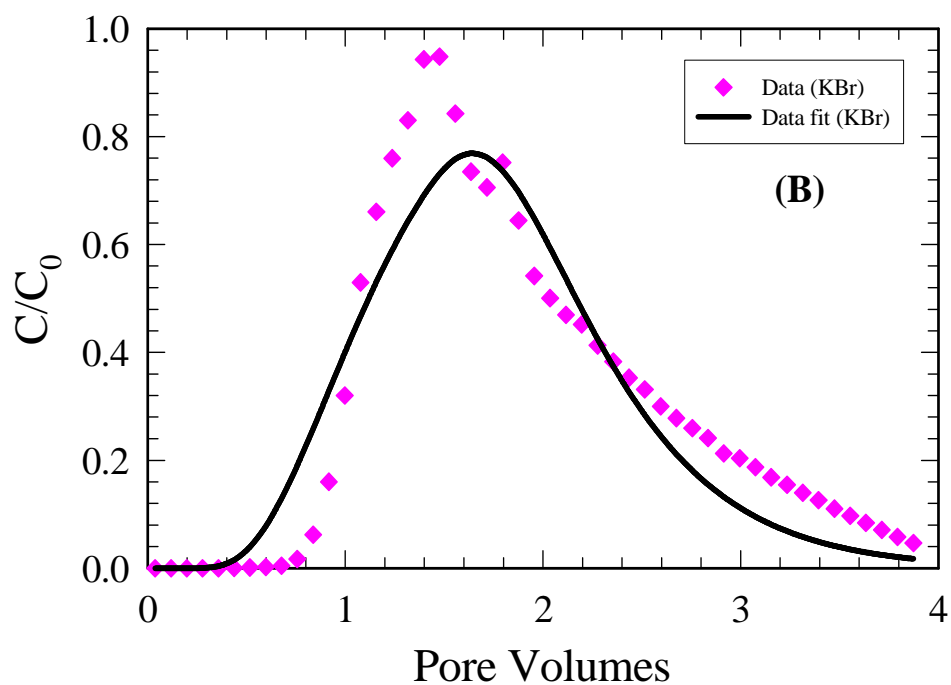
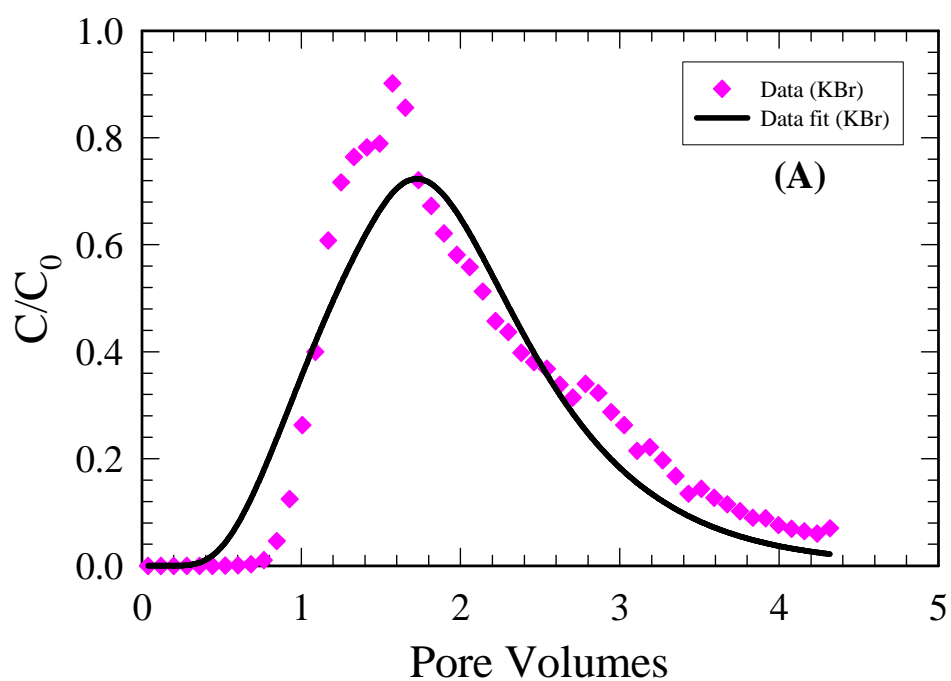


Figure A. Site map of Barksdale, WI.



## APPENDIX B

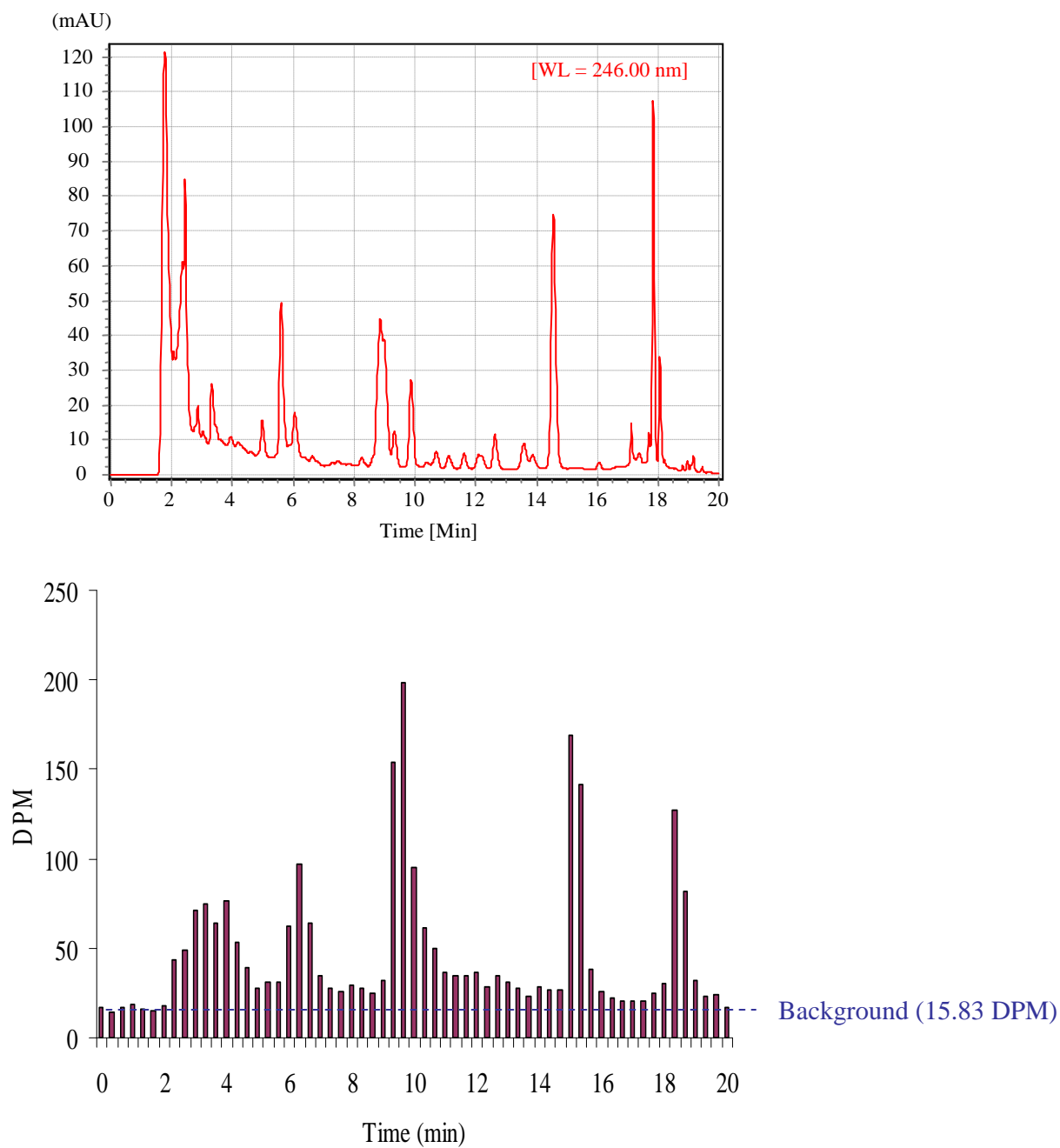
Appendix B (supplementary information for Chapter 6) shows the curve fitting results of BTCs based on the tracer analysis. The CXTFIT 2.0 program was used to fit KBr measured normalized concentration (*i.e.*,  $C/C_0$ ) versus cumulative pore volume data. Each data fit based on this program estimated the pore water velocity ( $v$ ) and dispersion coefficient ( $D$ ) listed in Table 6.4.



**Figure B.** Experimental data and model fitted breakthrough curves for KBr tracer (Data fit based on the CXTFIT 2.0 program), (A) 2,4-DNT degrading column; (B) 2,6-DNT degrading column.

## APPENDIX C

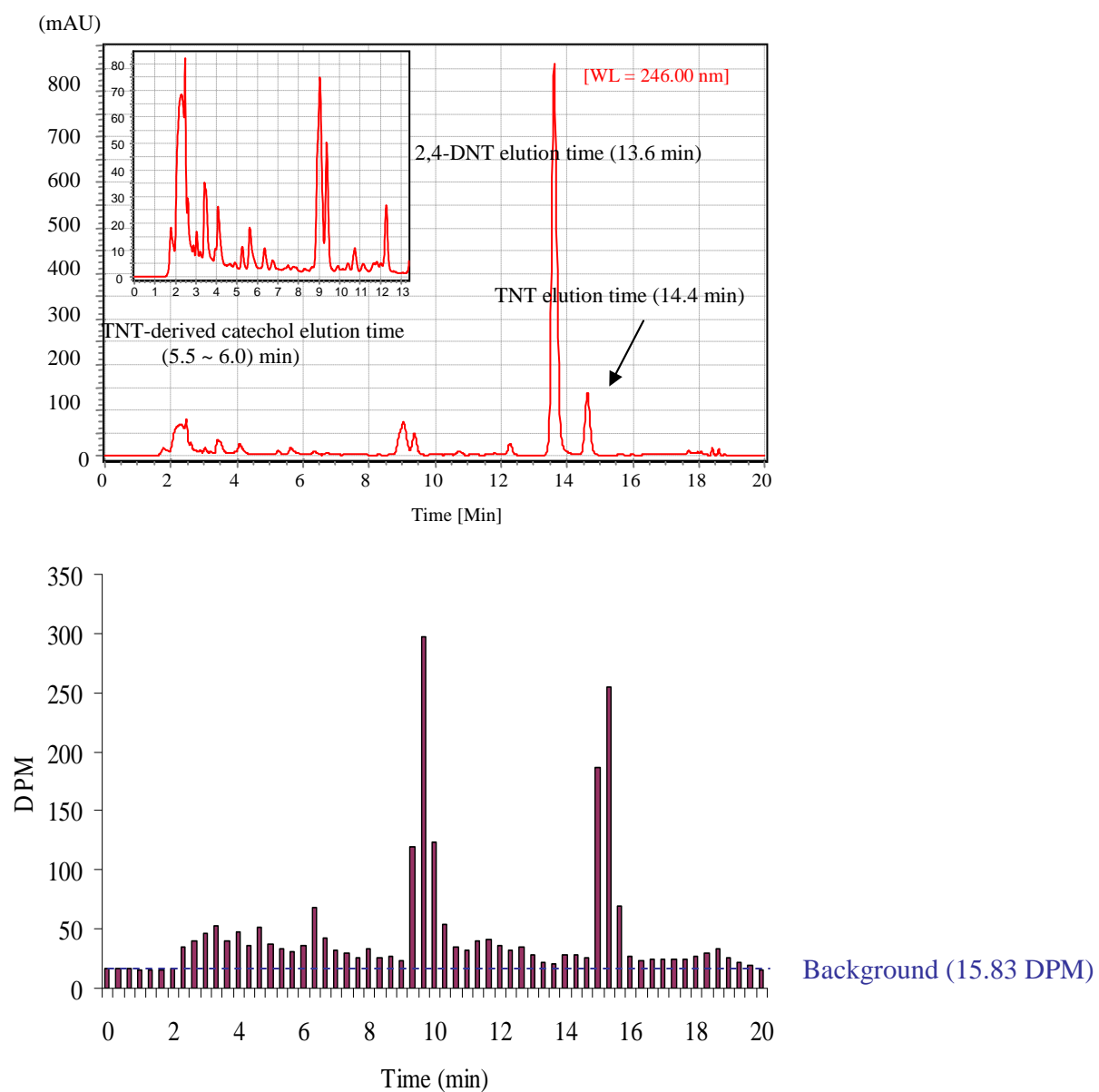
Appendix D contains the experimental data for the fate of depleted TNT in microcosm containing mixture of TNT and 2,4-DNT through separation and fractionation with end-point samples via HPLC. Similar to result from Figure 7.10, similar observations regarding oxidative/reduced metabolites and associated radioactive distribution were identified except portion of %  $^{14}\text{C}$  distribution in each fraction. These contain supplementary information for Chapter 7, entitled “Effect of mixtures of contaminants on degradation processes”.



**Figure C.** TNT cometabolism in the presence of 2,4-DNT. UV profile (Top) at  $\lambda = 246$  nm with corresponding  $^{14}\text{C}$  profile (Bottom) for microcosm sample containing both TNT and 2,4-DNT.

## APPENDIX D

This information pertains directly to Chapters 8 (TNT BioPilot studies). Included are separation and fractionation of  $^{14}\text{C}$ -labeled metabolites via HPLC from TNT/2,4-DNT mixture reactor. Similar observation was shown in both only TNT amended reactor (Figure 8.8) and TNT/2,4-DNT mixture reactor except the extent of %  $^{14}\text{C}$  distribution an each fraction.



**Figure D.** Extensive examination of aerobic TNT metabolism in the presence of 2,4-DNT. UV profile (Top) at  $\lambda = 246$  nm with corresponding  $^{14}\text{C}$  profile (Bottom) for microcosm sample containing both TNT and 2,4-DNT.